Universidade de Lisboa

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S100B targeting to reduce demyelination and EAE pathogenesis

Pedro Filipe Marques Pascoal

Dissertação de Mestrado orientada pela

Prof.ª Doutora Adelaide Maria Afonso Fernandes Borralho

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS

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The studies presented in this thesis were performed within the Neuron Glia Biology in Health and Disease research Group at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of de Lisbon, under the supervision of Adelaide M. A. Fernandes Borralho, PhD.

À minha mãe.

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Table of Contents

Figure Index

I. Introduction

II. Material and Methods

III. Results

Index of Tables

II. Material and Methods

Resumo

A esclerose múltipla (EM) é uma doença inflamatória crónica e autoimune com uma forte componente neurodegenerativa, resultando em défices severos nas funções motoras e cognitivas. Apesar da evolução do conhecimento nas últimas décadas, os mecanismos subjacentes à destruição da mielina e dos respetivos axónios, bem como a interação igualmente importante entre os mecanismos degenerativos e os mecanismos de reparação ainda não estão completamente esclarecidos. A proteína S100B é uma pequena molécula de ligação ao Ca^{2+}, sendo membro da família S100. Esta proteína é principalmente expressa por astrócitos e exerce funções quer intracelulares, atuando como molécula de sinalização, quer extracelulares, executando efeitos benéficos ou prejudiciais dependendo da sua concentração. Estudos recentes demonstraram que a expressão de S100B encontra-se aumentada em doentes com EM, e que os seus níveis elevados estão correlacionados com a reatividade da glia e o aumento da expressão do seu recetor RAGE, tendo um papel fundamental na resposta inflamatória exacerbada desta doença. Num trabalho mais recente o nosso grupo demonstrou o papel benéfico da inibição de S100B no decurso de um dano desmielinizante, usando um modelo *ex vivo.*

Com esse trabalho, pretendemos entender se ao modular a molécula inflamatória S100B, usando desta vez um novo composto, podemos prevenir a patogénese associada à doença de EM. Decidimos utilizar a pentamidina, fármaco correntemente utilizado na clinica para outras indicações terapêuticas, e que tem a capacidade de se ligar diretamente à S100B. Para abordar esta questão, primeiro fomos verificar se a ligação da pentamidina ao S100B poderia prevenir a patogénese associada ao modelo de desmielinização *ex vivo*. Os nossos resultados sugerem que a pentamidina é capaz de prevenir não só a desmielinização, bem como diminuir a produção exacerbada de fatores inflamatórios, indicando um efeito benéfico na prevenção da patogénese da desmielinização e da inflamação associada.

Em seguida, caracterizamos o eixo S100B-RAGE num modelo animal de EM, o Encefalomielite Autoimune Experimental (EAE), numa tentativa de perceber se seria um bom modelo para testar uma terapêutica dirigida para a S100B. Os nossos resultados mostraram que os animais induzidos para EAE

apresentam uma elevada expressão de S100B e RAGE, bem como citocinas pró-inflamatórias. Curiosamente, estes animais quando tratados com dimetil fumarato, um medicamento usado na prática clínica em EM, demonstraram uma reduzida expressão de ambas as proteínas S100B e RAGE em paralelo com a recuperação da patogénese desmielinizante.

Assim, não só comprovámos que o modelo de EAE é um bom modelo para ensaiar o novo medicamento pentamidina, como demonstrámos que a ligação S100B-RAGE pode ser uma nova estratégia terapêutica mais específica para prevenir a desmielinização e a expressão exacerbada de fatores inflamatórios.

Palavras-chave: Desmielinização, EAE, Esclerose Múltipla, pentamidina, RAGE, S100B.

Abstract

The autoimmune disease multiple sclerosis (MS) has strong inflammatory and neurodegenerative components, characterized by severe effects on motor and cognitive functions. Despite the scientific advances in this area in recent years, the mechanisms underlying myelin and axonal destruction and the equally important interaction between degenerative and repair mechanisms is not yet completely clarified. S100B is a small Ca^{2+} binding protein member of the S100 family. Is mostly expressed by astrocytes and can exert functions both intracellularly, acting as signaling molecule, and extracellularly, exerting beneficial or detrimental effects in a concentration-dependent manner. Recent studies demonstrated that S100B protein expression is increased in both CSF, serum and post-mortem plaques of MS patients and that its high levels are correlated with glial reactivity and increased expression of its receptor RAGE, having a role in the excessive inflammatory response of this disease. More recent work from our group has shown beneficial outcomes of S100B inhibition in an *ex vivo* demyelinating model.

With this work we intended to understand if by targeting the inflammatory molecule S100B with a specific drug pentamidine, already used in clinical practice for other therapeutic indications, we may prevent MS-associated pathogenesis. To address this issue, we first assayed whether the direct binding of the drug pentamidine to S100B could prevent the pathogenesis observed in the *ex vivo* demyelination model. Our results suggest that pentamidine is able to prevent demyelination and decrease the exacerbated production of inflammatory factors, indicating a beneficial effect in the prevention of demyelination and associated inflammatory pathogenesis.

Next, we also characterized the S100B-RAGE axis an *in vivo* animal model of MS, the Experimental Autoimmune Encephalomyelitis (EAE), as an attempt to identify a good animal model to assay the S100B-targeting therapy. Our results showed that EAE animals highly express S100B and RAGE, as well as other pro-inflammatory cytokines. Curiously, when the animals were treated with dimethyl fumarate, a drug used in clinical practice for MS, it was detected a decreased expression of both proteins in parallel to the reduced loss of myelin fibers.

Therefore, not only we demonstrated that EAE is a good model to assay the new drug pentamidine, but also showed that targeting S100B-RAGE axis may be a new and more specific therapeutic strategy to prevent demyelination and exacerbated expression of inflammatory factors.

Keywords: Demyelination, EAE, Multiple Sclerosis, pentamidine, RAGE, S100B.

Abbreviations

APC	Antigen-presenting cells
BBB	Blood-Brain-Barrier
Ca^{2+}	Calcium
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular patterns
DIV	Days in vitro
DMF	Dimethyl fumarate
EAE	Experimental autoimmune encephalomyelitis
EBSS	Earle's balanced salt solution
GFAP	Glial fibrillary acidic protein
HLA	Human leukocyte antigens
HMGB1	High-mobility group box 1
Iba-1	Ionized calcium-binding adapter molecule 1
IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Nitric oxide synthase
LPC	Lysophosphatidyl choline
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
OCSC	Organotypic cerebellar slice cultures

OL	Oligodendrocytes
OPC	Oligodendrocytes precursor cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PPMS	Primary progressive MS
PTX	Pertussis toxin
qRealTime-PCR	Quantitative real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RRMS	Relapsing-remitting MS
SPMS	Secundary-Progression MS
TCR	T-cell receptor
Th	T-helper
TLR	Toll-like receptor
TMEV-IDD	Theiler's murine encephalitis virus-induced demyelinating disease
TNF	Tumor necrosis factor
Treg	Regulatory T
WT	Wild-type

I. Introduction

1. Multiple Sclerosis: Overview

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disorder that affects the central nervous system (CNS). In this disorder focal lymphocytic infiltration across the Blood-Brain-Barrier (BBB), microglial activation and production of cytotoxic mediators leads to damage of myelin and axons. Consequently it occurs the development of acute focal and progressive demyelination with limited remyelination, leading to the formation of multiple sclerotic plaques in the brain and spinal cord (Lassmann 2010). The symptomatology can be very different, since it will depend on the CNS area where these plaques are formed (Lassmann et al. 2012, Friese et al. 2014). It is understood by demyelination the loss of myelin sheath covering the axons, compromising its efficiency on the correct signal transmission, leading to serious consequences at cognitive and motor functions (Love 2006).

The etiology of the disease is currently not yet clarified and only partly of its pathogenesis is understood, although with some controversy. Over the last years several authors have gathered strong evidence that it has an autoimmune pattern with auto-reactive immune cells crossing the BBB and attacking myelin and axons (Huseby ES 2001, Ransohoff et al. 2003, Perchellet et al. 2004).

The disease, although not considered to be life shortening, is one of the most common neurological disorders causing disability in young adults between 20 and 40 years old. Most recent data show that there are a total estimated number diagnosed patients of approximately 2.3 million around the world, with a prevalence of 33 per 100 000, being higher in developed regions (e.g. North

America and Europe, 140 and 108 per 100 000 respectively) than in underdeveloped countries (e.g. Sub-Saharan Africa and East Asia, at 2.1 and 2.2 per 100,000 respectively) (WHO 2013). It has been reported that the prevalence of MS varies according to latitude being associated with sun light exposure and vitamin D production (Evans et al. 2013). The impact on the quality of life of people with MS and the financial implications for society are therefore long lasting and profound (Jacquelyn L. Bainbridge 2007). Direct costs include inpatient care, outpatient care, costs for drugs, diagnostics, surgical interventions, nursing care, social services, as well as travel costs in order to get to health care. MS patient also have indirect costs that includes long-term and permanent reductions in productive work, resulting in early old-age pension due to health problems and short-term work absence (Ernstsson et al. 2016). In Portugal, the most recent study (de Sa et al. 2012) estimates that the prevalence is 56.20 per 100 000. This means, that in Portugal there are about 5620 MS patients per 10 million inhabitants.

1.1. Clinical course of the disease

There are two major forms of MS, Relapsing-remitting MS (RRMS) and primary progressive MS (PPMS) (Figure I. 1). In most patients, 85% to 90%, MS starts with RRMS and affects women about twice as often as men. This form is characterized by defined attacks of new or increasing neurologic symptoms (Compston and Coles 2008, Friese et al. 2014). These attacks, also called relapses, are normally followed by periods of partial or complete recovery, which are called remissions. During remissions, all symptoms may disappear, complete recovery, or continue and become permanent, disease worsening (Sospedra and Martin 2005). However, after 15-25 years, the relapses typically shift into inexorably progressive neurodegeneration, which is termed secondary progressive MS (SPMS) (McFarland 1999). Currently does not exist a clear clinical, imaging, immunologic, or pathologic criteria to determine this shift point when RRMS converts to SPMS, the transition is usually gradual (Lublin FD 2014).

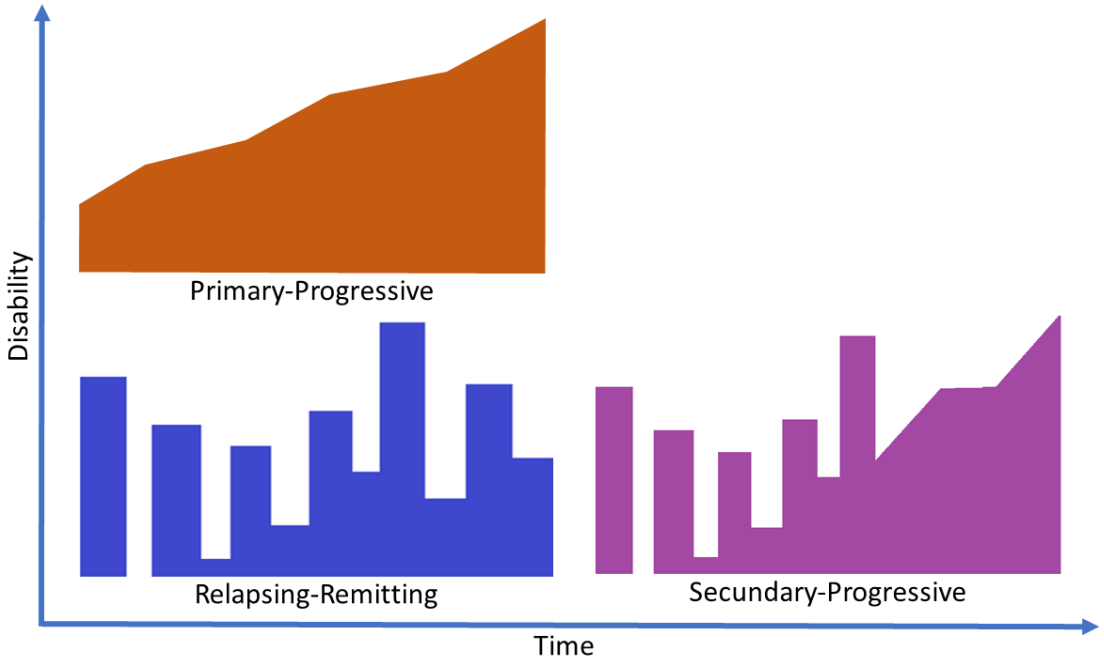

Figure I. 1 Schematic representation of the evolution of disability over time in different types of MS. Primary-progressive Multiple Sclerosis (PPMS) – Steady increase in disability without attacks; Relaping-Remitting MS (RRMS) – Unpredictable attacks wicth may or may not leave permanent deficits followed by periods of remission; Secundary-Progression MS (SPMS) – Initial RRMS that begins to have decline without periods of remission.

The second major form of MS, the PPMS, affects about 10%–15% of patients. PPMS is characterized by worsening of neurologic function (accumulation of disability) from the onset of symptoms, without early relapses or remissions (Sospedra and Martin 2005). It is not clear which factors are responsible for the different courses. However, it is thought that MS can be triggered by environmental factors in individuals with complex genetic-risk profiles that translates into different immune abnormalities and increased vulnerability of CNS tissue to inflammatory insult or reduced ability to repair damage (Gregory et al. 2007, Duarte et al. 2012).

Focal plaques of demyelination are the diagnostic hallmark in MS pathology and they can be distinguished in different types according to how they progress: active, chronic active, inactive, and remyelinated shadow plaques. The classic active plaques are characterized by a perivascular Infiltration of lymphocytes and macrophages together with a high inflammation, the remyelination is impaired as long as inflammation is active. The chronic active lesions are characterized by having no myelin or remyelination signals in the center, being surrounded by an area of microglial activation. The inactive lesions are the most frequent type in all stages of the disease, these lesions are the result of the demyelination and

axonal loss that have an inefficient remyelination. This state is also characterized by the reduced lymphocytic infiltrates and microglial activation, having no demyelinating or neurodegenerative activity at the lesion border. Lastly the shadow plaques present widespread stable remyelination and the inflammation has subsided to normal levels (Love 2006, Compston and Coles 2008, Lassmann 2010, Lassmann et al. 2012).

1.2. Etiology and pathogenesis in MS

The etiology of MS is not fully understood yet, but typically this disease pursues a relapsing and remitting course that becomes progressive after initial remissions, that leads to the formation of focal plaques, accumulating irreversible neurological deficits. However, it can be progressive from the outset. These Relapses result from inflammatory demyelinating lesions in the CNS and have no specific pattern in the intervals between them. It is thought to be caused by the interaction of multiple genetic and environmental factors (Love 2006, Compston and Coles 2008). The clinical manifestations of MS are wide ranging, generally involves the motor, sensory, visual and autonomic systems in which we can include weakness, paraesthesia or focal sensory loss, optic neuritis, diplopia, ataxia and vertigo, but many other symptoms and signs can occur (Charil and Filippi 2007, Compston and Coles 2008).

The mechanisms underlying myelin and axonal destruction and the equally important interaction between degenerative and repair mechanisms is still widely discussed. In an early phase of the disease the inflammation caused or aggravated by auto-reactive immune cells that cross the BBB attacking myelin and axons is followed by remyelination that lead to a total or partial clinical remission of the symptomatology. The problem seat on the repeated acute insults after the first relapse, together with consequent inflammation. Remyelination and the reparative system in general fails leading to an extensive microglial activation over time, resulting in an irreversible axonal degeneration and neuronal death (Charil and Filippi 2007, Glass et al. 2010). Therefore, early diagnosis is of great importance. Over the last years, the ability to diagnose MS early in the disease course has been refined with the use of magnetic resonance imaging (MRI), however the clinical features of the patient needs to be considered to obtain an accurate diagnosis, depending too much on the imaging can lead to errors of

diagnosis when these clinical characteristics are absent (Solomon and Corboy 2017). Current medical treatment targets the immune system in an attempt to delay the progression of the disease without actually curing the disease. In order to obtain a fastest MS diagnosis, is necessary to identify specific and more sensitive biomarker that distinguishes MS from other diseases as well as new therapeutic targets that can that take us a step further in the search for the cure of this disease.

1.2.1. Central and peripheral immune system

It is well accepted that the CNS is considered an immune-privileged site. The brain monitors and coordinates internal organ function and responds to changes in the environment, so, as a main system, it needs to be protected from the entry of pathogens, circulating immune cells, and factors within the blood (Louveau et al. 2015). One of the main defenses is a physical BBB, formed by brain endothelial cells, astrocytes and various proteins that prevents potentially damaging agents from accessing the CNS (Takeshita and Ransohoff 2012). However, the meninges, set of three membranes that envelop the brain and spinal cord, are more accessible to these agents, being thus populated by various immune cells (Decimo et al. 2012). The mechanism by which these cells and antigens from the CNS trigger immune responses has remained unclear (Kipnis et al. 2012). Unlike the peripheral immune system, in the brain there is an absence of professional antigen-presenting cells (APCs), besides microglia, there is a low major histocompatibility complex (MHC) class I and II expression. In addition, it also seems to have a relative lack of lymphatic drainage of the parenchyma, that clearly could be limiting the ability to handle with CNS derived antigens, and the exchange of immune cells and mediators (Louveau et al. 2015). Despite all of this, interactions between the CNS and the immune system occur, resulting in the maintenance of proper brain homeostasis (Choi and Kim 2008). Unfortunately as has been suggested, a BBB dysfunction can precede immune infiltration that incite demyelination in MS (Larochelle et al. 2011). Autoreactive T-cells cross the BBB and are activated locally by microglia and astrocytes. The resulting secretion of pro-inflammatory molecules from these T cells, which include cytokines and chemokines, increases the permeability of the barrier, leading to increased recruitment of other immune cells (McQualter and Bernard

2007). These inflammatory infiltrates are composed by macrophages and lymphocytes, including natural killer cells (NK cells), T cells and B cells (Hauser et al. 1986, Lassmann and van Horssen 2011).

Macrophages are professional phagocytes and are highly specialized in removal of dying or dead cells and cellular debris (Gregory and Pound 2010). This role is important in chronic inflammation, as the early stages of inflammation are dominated by neutrophils, which are ingested by macrophages if they come of age (Han et al. 2016). The macrophage phenotype spectrum is characterized by two main groups designated by classical M1 macrophages and alternative M2 macrophages (Mantovani et al. 2009). M1 is induced by pro-inflammatory mediators released by T-helper 1 (Th-1), such as interferon-γ (IFNγ), tumor necrosis factor (TNF)-α and Toll-like receptor(TLR)-4, being characterized by the production of numerous pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and TNF-α (Mosser 2003, Verreck et al. 2004, Colin et al. 2014). On the other hand, the alternative M2 macrophage is induced by anti-inflammatory mediators such as IL-4 and IL-13 cytokines, produced by Th2 cells, and IL-10 (Gordon 2003). These M2 macrophages, mainly involved in tissue repair, display phagocytic, pro-angiogenic, and profibrotic capacities (Mosser 2003).

As mentioned above, in addition to macrophages, lymphocytes also play a critical role in the inflammatory response present within the demyelinating lesions. The vast majority of them are MHC Class I restricted $CD8^+$ T cells (Babbe et al. 2000), while MHC Class II restricted CD4+ T cells as well as B cells are mainly seen in perivascular spaces and in the meninges (Lassmann and van Horssen 2011). The activation of $CD4^+$ autoreactive T cells and their differentiation into a Th1 phenotype have a key role in the initial steps of the disease, it is thought that these cells are also important players in the evolution of the disease. On the other hand, the damage of the CNS is, most likely mediated by the $CD8^+$ T cells, and other components of the immune system such as complement, antibodies and factors produced by innate immune cells. Regulatory T (Treg) cells, are a specific suppressor subtype of $CD4^+$ T cells and they are vital for the maintenance of dominant self-tolerance and immune homeostasis. Treg cells can be differentiated in the thymus during the T cell

development (natural Treg), or induced by peripheral regulatory mechanisms in the peripheral immune system organs (inducible Treg) (Sakaguchi et al. 2010, Jamshidian et al. 2013). Treg have been shown to limit the pathology or to prevent its onset in MS (McGeachy et al. 2005, Awasthi et al. 2008). Recently another effector subset of CD4+ T cells was discovered, Th17, which is characterized by IL-17A cytokine production, but unlike Tregs, stimulates a range of inflammatory mediators including TNF, IL-9, IL-17A, IL-17F, IL-21, IL-22, showing an important pro-inflammatory role in the immune system. If these cells are not efficiently controlled by regulators, they can promote many autoimmune inflammatory conditions (Wilson et al. 2007). Under certain *in vitro* inflammatory conditions, Treg cells have a tendency to be reprogrammed and converted to the inflammatory Th17 subset (Koenen et al. 2008). The Treg/Th17 balance has been shown to be similarly disturbed in other autoimmune and inflammatory conditions, with decreasing Tregs versus increasing Th17 cells (Liu et al. 2011, Shen et al. 2011). Therefore, Treg/Th17 balance, has a key role in the regulation of inflammatory reactions and is an imperative checkpoint in immune homeostasis.

1.2.2. Central nervous system demyelination

Oligodendrocytes (OL) are the myelinating cells of the CNS. The myelin membrane that involve the axon provide them protection and nutritional support (Mei et al. 2013), as well as improves the conduction of action potentials, being of critical importance for neuronal survival and integrity (Franklin and Ffrench-Constant 2008). A single OL can extend processes to numerous axons (McTigue and Tripathi 2008). In order to properly myelinate, OL have to undergo many changes at morphological and specific protein expression levels that will allow the contact and enwrapping of a stretch of axon. Four transitional cell stages have been described: OL precursor cells (OPC), preoligodendrocytes (or late OPC), immature (or pre-myelinating) OL and mature (or myelinating) OL (Levine and Reynolds 1999). OL maturation is driven by multiple signals that regulate their migration, proliferation and differentiation. OPC are characterized by expressing platelet derived growth factor receptor (PDGFR)α and the proteoglycan neural glial antigen 2 (NG2) having few and short multipolar ramifications. On the other hand, pre-myelinated OL cells, present long and ramified branches that will be extended towards multiple axons, initiating the expression of myelin proteins.

When this contact is established, mature OL start expressing myelin proteolipid protein (PLP) and myelin basic protein (MBP), forming membranes that enwrap the axons forming the myelin sheath.

As mentioned above, it has been suggested that demyelination is triggered by autoreactive T-cells that cross the BBB and activate local cellular elements. Once set, immune activation and CNS inflammation generates a multitude of specific and non-specific mechanisms of OL and myelin injury (Steinman 1996). Consequently, OL cell death and disruption of myelin integrity eventually led to tissue disintegration (Hafler 2004).

Despite the destruction of myelin sheaths, some OL may survive the initial inflammatory insult, however, with repeated insults even surviving OL are destroyed. Theses chronically recurrent episodes of inflammatory demyelination result in the relapsing pattern of the disease (Smith and McDonald 1999). Furthermore, OL replacement from a progenitor pool usually take place in an attempt to myelin repair (Wolswijk 2002). However, remyelination generally fails to achieve complete repair, mainly because of the reduced ability of OPC to proliferate and differentiate into mature OL, resulting in an irreversible axonal degeneration and neuronal death (Glass et al. 2010).

1.2.3. Glia reactivity

Following CNS injury, an intensive local inflammatory response takes place and involves the activation of the resident microglia, the native macrophages of the CNS (Xu et al. 2015). These cells modulate neuronal function not only during an inflammatory response but also during developmental synaptic pruning (Paolicelli et al. 2011) and plasticity in the healthy brain (Parkhurst et al. 2013), being able to rapidly respond to even minor changes in the CNS.

In normal situations, microglia have a ramified morphology, characterized by little perinuclear cytoplasm, and a number of thin branched processes, being able to search the local environment for infectious agents or harmful material (Ransohoff and Perry 2009). Microglia also monitor the functional state of synapses, eliminating synaptic elements by phagocytosis and remodeling extracellular spaces (Kettenmann et al. 2013, Brown and Neher 2014). During an inflammatory response to a harmful stimulus, microglia became activated and rapidly switch their morphology from ramified to a more amoeboid shape, with

thicker and retracted processes (Cho et al. 2006, Taylor et al. 2014), releasing neurotrophic factors to promote neuron growth and migration (Kettenmann et al. 2011, Greter and Merad 2012), and recruitment of monocytes from the peripheral blood (McQualter and Bernard 2007). Activated microglia migrate to the site of injury, secrete proteases that promote microglia mobility, and then they participate in the phagocytosis of cell debris, as well as in tissue repair by secreting growth factors and other molecules, which modulate extracellular matrix (Kreutzberg 1996, Hanisch and Kettenmann 2007, Ransohoff and Perry 2009). Most importantly, microglia produce pro-inflammatory cytokines or reactive oxygen species (ROS) to attack the pathogen and are crucial for antigen presentation by expressing cell surface antigens and several immune receptors. One of them is Iba-1, which has been widely used in the study of microglia due to its specific expression in microglia/macrophages and because it detects both reactive and ramified microglia. If the damage is excessive, microglia continue to change their morphology and adopt an amoeboid shape, to the point where they become indistinguishable from blood-borne macrophages (Ransohoff and Perry 2009, Taylor et al. 2014). Lastly, if the inflammation became chronic microglia assume a hypertrophic branch morphology with an enlarged soma (Loane et al. 2014).

Similar to macrophages, microglia can also develop a range of phenotypes that broadly corresponds to these discussed above. The classically activated (M1) and an alternative activated (M2) phenotype (Colton 2009, Olah et al. 2011, Correale 2014). M1 consists of a pro-inflammatory phenotype and is responsible for mediating innate immune responses and adaptive immunity, being the initial source of pro-inflammatory cytokines, such as TNF-α, IL-23, IL-1β and IL-6, proteases and ROS (Crain et al. 2013). These responses may be induced by damage-associated molecular patterns (DAMPs) and other proteins released from injured neurons. The high-mobility group box 1 (HMGB1), is a DAMP that can be secreted by astrocytes, microglia and damage neurons (Andersson et al. 2008). HMGB1 can triggers inflammatory response by interacting with TLRs, but mainly the interaction occurs with receptor for advanced glycation end products (RAGE), inducing production of other pro-inflammatory cytokines in myeloid cells

(IL-1, TNF-a, IL-6, IL-8) and further activate microglia, exacerbating the nuclear factor kappa-B (NF-kB) signaling cascade (Kokkola 2005, Brites and Vaz 2014).

On the other hand, M2 microglia promote phagocytosis of cellular and myelin debris contributing for the initiation of remyelination process. This phenotype is induced by the cytokines IL-4 or IL-13 released from Th2 cells and associated with the production of anti-inflammatory cytokines and trophic factors, playing an important role in tissue repair, contributing to neuroprotection (Zhao et al. 2006). M2 microglia can be subdivided in three subclasses depending on their functional properties. M2a, induced by IL-4 or IL-13, is responsible for phagocytosis and inflammation repair (Correale 2014). Immune-regulatory, M2b phenotype, is induced by immune complexes and TLR agonists, and is associated with increased neuronal loss (Chhor et al. 2013). Acquired deactivation, M2c phenotype is produced by inducing agents like transforming growth factor (TGF)-β and IL-10, and associated with a robust suppression of the innate immune system, mainly through an autocrine upregulation of the anti-inflammatory cytokine IL-10 (Colton 2009).

The role of microglia in MS is still controversial. As mentioned above, it has been widely accepted that the disease process is initiated when autoreactive T-cells cross the BBB and are activated locally by microglia and astrocytes leading to inflammation and demyelination. However, some researchers have also found that OL apoptosis in MS lesions is not associated with T cells or peripheral macrophages (Henderson et al. 2009). On the other hand, microglial activation has been observed in post mortem brain tissue of MS patients (Lucchinetti et al. 2011, Giunti et al. 2014). Indeed, our group also observed a large population of activated microglia in active MS lesions (Barateiro et al. 2016). Several studies have associate the microglia M1 phenotype with neuroinflammation and demyelination in MS by showing that the inhibition of microglial activation decrease the onset of the disease in a demyelination mouse model (Bogie et al. 2014, Giunti et al. 2014). However, for remyelination processes to occur, is essential that microglia switch their phenotype from M1 to M2, secreting neurotrophic molecules that will mediate the recruitment of OPC to the demyelinated foci (Miron et al. 2013). Since microglial cells present distinct role along disease progression, it is of great interest to understand and modulate the

reactive role that these cells have in MS into a more neuroprotective phenotype during the course of the disease.

Astrocytes are the most abundant non-neuronal cell type within the brain. They are active players in mechanisms of synaptic transmission and plasticity, being fundamental in the regulation of cerebral metabolism (Belanger et al. 2011). Glycogen, as an important energy source for the brain, is stored mainly in astrocytes, that provide energy substrates in the form of lactate, when higher dose of energy is needed to neurons (Allaman et al. 2011). In addition astrocytes have also an important role in several other cerebral functions, such as the regulation of extracellular glutamate levels, modulation of synaptic activity, disposal of neuronal catabolic products, control of ion and water homeostasis (Gabriel et al. 2004), promotion of the myelinating activity of OL (Lutz et al. 2009) and defense against oxidative stress (Finsterwald et al. 2015). However, the precise role of astrocytes in regulating all of these functions remains unclear.

Similar to microglia, astrocytes have numerous processes to sense their surrounding environment, being able to respond to extracellular changes (Belanger et al. 2011). Some of these processes lie close to synapses, whereas others are in contact with brain capillaries, being enclosed with all cellular components of the CNS. Astrocytes are also a source of key regulatory factors such as TGF-β, glial-derived neurotropic factor, and the fibroblast growth factor (Dohgu et al. 2005). Their morphology varies between brain areas (Bernardinelli et al. 2014) and changes dramatically during disease or injury, when become reactive (Sun and Jakobs 2012). Theses reactive astrocytes are generally identified by upregulation of glial fibrillary acidic protein (GFAP), the prototypical marker for immuno-histochemical identification of these cells within the CNS (Sun and Jakobs 2012). Reactive astrocytes can repair tissues (Chien et al. 2014), by releasing diverse pro- and anti-inflammatory cytokines, chemokines and neurotrophins. However, a disturbance during the process of reactive astrogliosis, can potential underlie neural dysfunction pathology including in MS (Sofroniew and Vinters 2010). Indeed, astrocytes were found to be highly abnormal, early in the study of MS lesions (Correale 2014). Furthermore, studies in experimental autoimmune encephalomyelitis (EAE) have shown that activation of astrocytes, and its consequential loss of processes around small blood vessels

may be linked to early loss of BBB function (Brosnan and Raine 2013). Astrocytes are important components of the BBB, crucial for its formation and maintenance (Cheslow and Alvarez 2016), being able to influence particular BBB features such as permeability, leading to tight junction formation and expression of related proteins in endothelial cells (Alvarez et al. 2011). It is thought that activated astrocytes can release some factors that may either suppress or promote the loss of barrier properties in the inflamed CNS (Ludwin et al. 2016). It has been documented that in most demyelinated areas, activated astrocytes secrete compounds with toxic effects for neurons, namely axons, and OL, having a consequent impact on myelin (Brosnan and Raine 2013).

1.3. Current therapeutic strategies for Multiple Sclerosis

There is currently no cure for MS. However, there is strategies to modify the course of the disease. Although there is a lack of consensus, our broad understanding of MS immunopathogenesis continues to promote clinical research. As such, several new treatment trials examining promising therapies are under development and emerging therapeutic targets continue to be identified. In the meantime, optimizing our current disease modifying therapies (DMTs), that have been found to slow disease progression and prevent disability symptoms, may also help the disease. The major goals in MS therapy are to reverse neurological deficits that occur during relapses and prevent relapses and disease progression. Therefore, it is of great importance to work early in disease management, in order to intervene prior to irreversible neuronal destruction, delay disability progression and improve quality of life. There are now 11 medications that have been approved by US FDA as disease modifying agents (DMAs) for relapsing forms of MS (Loleit et al. 2014). The latest addition, dimethyl fumarate (DMF), was approved by the FDA in March 2013. The mode of action of this drug is not fully understood, but, recent studies shown that DMF particularly targets proinflammatory mature B cell subsets through preferential induction of apoptotic cell death as well as downregulation of NF-kB in surviving B cells (Li et al. 2017). Another drug that has been commonly used for MS treatment is the Natalizumab, a humanized monoclonal antibody against the cell adhesion molecule α4-integrin. By blocking these interactions, natalizumab interferes with the migration of lymphocytes across the BBB and thus reduces

inflammation in the CNS (Yednock et al. 1992). Fingolimod, is another drug among the most used to MS treatment. It is an immunomodulating drug that reduces the rate of relapses in RRMS, by sequestering lymphocytes in lymph nodes, preventing them from contributing to an autoimmune reaction (Brinkmann et al. 2010). Every treatment has its specific beneficial and adverse effects but the more effective drugs are associated with more severe side effects. Therefore, further studies including combination therapies and new and more specific therapeutic targets are needed to find most promising treatments.

2. S100B

S100B is a small binding protein containing two EF-hand helix-loop-helix Ca^{2+} binding domains, being one of the largest proteins of the S100 family involved in Ca^{2+} homeostasis (Kawasaki et al. 1998). S100B is mainly expressed by astrocytes, Schwann cells and a small subset of OL in the CNS (Hachem et al. 2005, Donato et al. 2009). Besides the CNS, S100B expression has also been found in adipocytes, lymphocytes, cardiomyocytes, bone marrow cells, chondrocytes and melanocytes. In these cells, S100B may be observed both in a soluble form diffusely expressed in the cytoplasm or associated with intracellular membranes, exerting both intracellular and extracellular functions (Donato et al. 2009). S100B take part in the regulation of Ca^{2+} homeostasis, protein phosphorylation, transcription, enzyme activity and metabolism. It also acts as a stimulator of proliferation and migration as well as an inhibitor of apoptosis and differentiation (Sorci et al. 2013). Moreover S100B, plays a critical role during brain development acting as a neurotrophic factor, by fostering neurite outgrowth and neuronal survival (Donato et al. 2013).

2.1. Dual role of S100B in CNS physiology and pathology

As mentioned above, S100B can exert both intracellular and extracellular functions. Intracellularly, this protein acts as a signaling molecule being involved in cell proliferation, migration and shape, Ca^{2+} homeostasis, protein phosphorylation, membrane trafficking, transcription, cytoskeleton dynamics as well as enzyme activity and metabolism. S100B also act as an inhibitor of apoptosis and differentiation; being also involved in the stimulation of astrocyte and microglia activation, which may have implications for brain repair after CNS

injury, namely following MS-associated neuroinflammation (Donato et al. 2009). Therefore, it is clear that S100B plays a protective role as long as it is maintained intracellularly at physiological levels.

As an extracellular factor, S100B can exert a dual effect depending on the concentration released and the microenvironment, being neurotrophic at low concentrations and neurotoxic at high concentrations (Donato 2007). At physiological concentrations S100B behaves as a signaling trophic protein promoting neurite extension and neuronal survival, enhancing astrocytic proliferation, and promoting microglia chemotactic ability (Selinfreund et al. 1991, Reali et al. 2005, Zhang et al. 2011). On the other hand, under stress conditions, like neuroinflammation (Ondruschka et al. 2013), S100B reaches high concentrations and exerts neurotoxic effects on neurons, with production of ROS. In addition, inflammatory activities of astrocytes are potentiated and microglia is activated, releasing inflammatory and oxidative stress mediators, contributing even more to neuronal cell death (Adami et al. 2001, Donato et al. 2009, Sorci et al. 2010).

Consistent with the notion that S100B can activate RAGE and that RAGE is expressed in developing neurons (Hori et al. 1995), several studies have shown that this double role of extracellular S100B are mediated via RAGE engagement (Bianchi et al. 2007, Donato 2007, Tsoporis et al. 2010). Its binding and effect is dependent on the intensity and extent of activation of the receptor, as well as the concentration of extracellular S100B (Donato 2007).

Results from Hofmann et al. (1999) have suggested that active S100B release might be dependent on the presence and activation of RAGE on the cell surface, and that S100B might stimulate its own release via RAGE engagement, which was further corroborated by more recent studies (Perrone et al. 2008, Donato et al. 2009, Sorci et al. 2013).

RAGE is a multiligand receptor of the immunoglobulin-superfamily, that interacts with multiple ligands, including S100B, mediating diverse functions as cellular migration and proliferation, survival and apoptosis (Donato et al. 2013). RAGE is primarily involved in inflammation, nephropathy, neurodegeneration, and cancer, and while in most tissues is expressed at low levels, at pathologic sites is usually upregulated (Ramasamy et al. 2007).

Over the last years S100B has gained relevance in numerous studies, given its involvement in several cellular events and important functions as described above, such as regulation of cell proliferation and differentiation, as well as the control of the assembly of cytoskeleton components, intracellular Ca^{2+} homeostasis and protein activities (Donato et al. 2009), being present in several CNS cells (Fig. I.2).

In astrocytes, S100B is involved in the assembly of cytoskeleton components important for maintaining their morphology and is also involved in their migration via interaction with Src kinase by stimulating the phosphatidylinositol 3-kinase (PI3K) pathways, a family of enzymes involved in cellular functions such as cell growth, proliferation and differentiation (Brozzi et al. 2009, Donato et al. 2009). Regarding microglia, S100B facilitate their migration and can be implicated on the regulation of microglial activities such as phagocytic ability by acting both inside and outside of the cell, that is corroborate by Adami et al. (2001) who found that S100B is associated with microtubule-like structures and centrosomes on microglia. On neurons, S100B seems to play an important role in the regulation of their proliferation and differentiation during early development stages (Arcuri et al. 2005). At last, S100B also have some functions on OL, particularly along their differentiation and maturation, being mostly expressed between non-myelinating pre-oligodendrocytes and mature OL, suggesting its involvement in their proliferation and differentiation (Deloulme et al. 2004).

2.2. S100B in MS

In case of damage or necrosis, astrocytes are known to be the main cell type in CNS that express and secrete S100B. This molecule may be considered a biomarker of brain damage, since its high concentration is found in several CNS pathologies (Donato et al. 2013). In MS, S100B elevated levels were first detected in the cerebrospinal fluid (CSF) of patients in an acute phase of the disease by Michetti et al. (1979). More recently, our group confirmed a significant increase of S100B production in CSF and serum at the time of diagnosis in RRMS patients being overexpressed in active and chronic lesions mainly by reactive astrocytes (Barateiro et al. 2016). We also observed an increase of RAGE expression co-localized with microglia/macrophages cells in active lesions. As

mentioned above, RAGE engagement mediates several of the detrimental effects resulting from S100B overexpression, moreover, this receptor is mainly expressed by microglia, so the interaction resulting from S100B/RAGE, can have a key role in the course of MS (Bianchi et al. 2011, Donato et al. 2013). It is therefore, of great interest to explore its role in brain damage related with demyelinating and inflammatory pathologies.

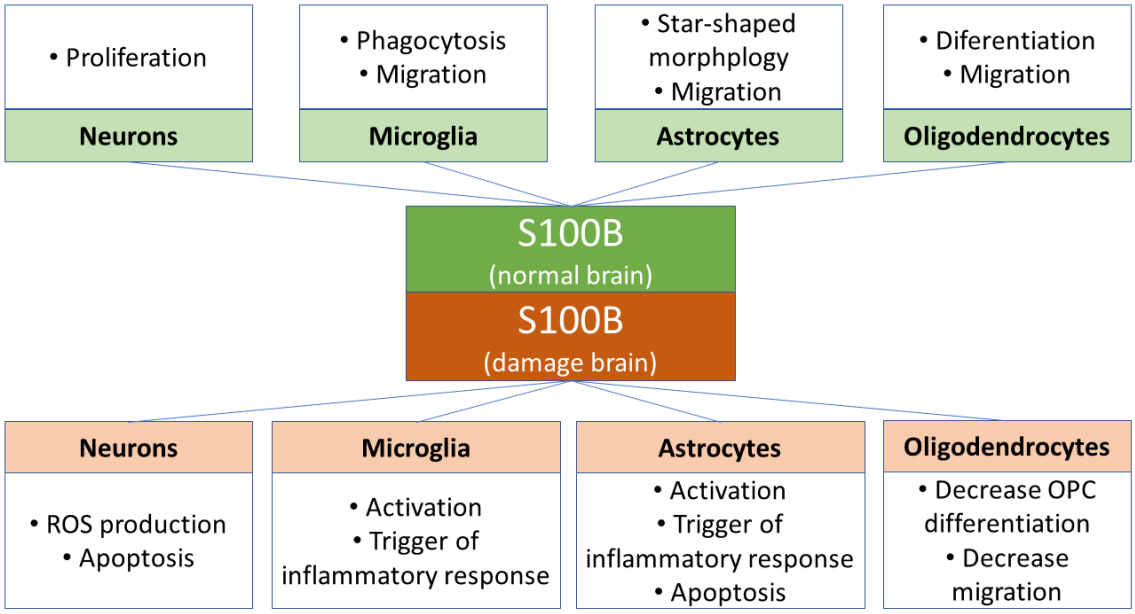

Figure I. 2 – Schematic representation of S100B effects in healthy and damage CNS. At physiological concentrations S100B has a neurotrophic effect, while under stress conditions, reaches high concentration and has a neurotoxic effect over neurons and glia cells.

As mention above, low S100B concentrations have a neurotrophic effect on neurons activating anti-apoptotic pathways via RAGE engagement as well as stimulating the extracellular signal-regulated kinases (ERK) of NF-kB increasing neuronal survival during injury (Selinfreund et al. 1991) (Huttunen et al. 2000). However, under stress conditions, S100B reaches high concentration and has a neurotoxic effect via overproduction of ROS and activation of apoptotic pathways (Donato et al. 2009). This neurotoxic effect of S100B also affects microglia, astrocytes and OL (Fig. I.2). High S100B levels in cooperation with IFN-γ may mediate microglia activation, triggering the brain inflammatory response (Adami et al. 2001), Similar, on astrocytes, high S100B levels stimulate the release of IL-6 and TNF-α and trigger the inflammatory response, furthermore, this high concentration of S100B also induce nitric oxide synthase (iNOS) activity via NF-kB, leading to astrocyte apoptosis (Brozzi et al. 2009).

2.3. Therapeutic Strategies for the S100B

The importance of S100B in MS has been the focus of our study because of its increased expression during MS episodes. Together with its involvement in other neurodegenerative diseases and its role in astrocytes and microglia activation, this protein seems to be a valuable therapeutic target that may possible be used to change the reactivity of glial cells to a more neuroprotective phenotype during the course of MS.

Several attempts have been made to lower the concentration of S100B in a direct manner. In a recent work, Hao et al. (2017) succeeded to prevent developmental upregulation of S100B expression using arundic acid in an embryonic gut culture system. Their results showed that preventing S100B synthesis, inhibited the development of the enteric nervous system by altering the levels of Sox10 protein in early differentiating glia, which is vital for maintaining their proliferative potential. Further, others studies also reported that arundic acid negatively regulates reactive astrocyte-associated S100B levels in a transgenic mouse model of AD-like cerebral amyloidosis (Mori et al. 2006). In another work, this agent was also shown to inhibit both the astrocytic overexpression of S100B and the subsequent activation of signaling pathways in the peri-infarct area in a rodent ischemia model (Asano et al. 2005). These results suggest that pharmacological inhibition of S100B biosynthesis may be a novel and valuable therapeutic target.

Therapeutic antibody-mediated neutralization of S100B is another approach to lower the concentration of free S100B. Using an experimental demyelinating *ex vivo* model, our group demonstrated that lysophosphatidyl choline (LPC)-induced demyelination upregulates S100B as well as astrocytosis, microgliosis and enhances the expression of several important pro-inflammatory cytokines. Most attractively, group results also showed that the neutralization of S100B using a specific antibody prevented LPC-induced demyelination, reactive astrogliosis, cytokine and inflammasome markers expression (Barateiro et al. 2016).

Recent studies with promising results, pointed pentamidine, an ancient antiprotozoal drug, as a potential small molecule that directly binds S100B blocking intracellular S100B interaction with the tumor suppressor p53 and

leading to downregulation of S100B and RAGE expression (Cirillo et al. 2015). Interestingly, their results in a mouse model of amyloid-β-induced Alzheimer's disease showed a consequent reduction of pro-inflammatory mediators and markers, preventing gliosis and neuroinflammation associated with amyloid-β.

In an indirect manner, other potential therapeutic strategies may be the use of specific RAGE antibodies or antagonists to prevent S100B binding. Indeed, RAGE specific antibody, was shown to control disease progression in a mouse model of Alzheimer's disease (Deane et al. 2012), while our group have described a decreased demyelination and inflammation in our *ex vivo* demyelinating model following the use of RAGE antagonist FPS-ZM1 (Santos et al. 2017). In another study, was shown that blockade of RAGE using soluble RAGE (sRAGE) cause a sharp decrease of cellular infiltration of the CNS by immune and inflammatory cells in EAE-induced animals, they observed the same effects when EAE occurred spontaneously in T-cell receptor (TCR)-transgenic mice. Furthermore, they also report that when fully activated 1AE10 cells were transferred into their mice, induction of EAE was also prevented by RAGE blockade in parallel with decreased entry of 1AE10 cells into the CNS. Therefore, targeting RAGE holds also a great potential to control the effect of extracellular concentration of S100B (Yan et al. 2003).

All these therapeutic strategies that act directly on S100B protein or indirectly by targeting RAGE receptor, have shown promising results during the last years in several CNS disorders, being able to reduce the inflammatory response during a demyelinating insult. So, their use to target MS may open new avenues for therapeutic intervention to reduce damage and improve recovery.

3. Experimental Demyelinating Models

The therapeutic inefficacy of current drugs and our poor understanding of the neurodegenerative mechanisms involved in MS, led to the development of experimental models that mimic both the symptoms and the hallmarks of the disease. Over the past several decades, a number of animal models have been developed in order to understand a variety of aspects of human MS, mainly this models are rodents, including rat and mice because of their relative genetically closeness to us, humans, but also because they are easy to manipulate (Denic

et al. 2011). Besides these *in vivo* models, it is possible to study demyelination and remyelination using *ex vivo* models. For decades, organotypic slice cultures, were used in research as an *ex vivo* model, due to the maintenance of a three-dimensional architecture and the contact between all different CNS cells that are important and play a key role in MS lesions (Kipp et al. 2012, Madill et al. 2016). In MS research, these cultures represent a model of intermediate complexity between *in vitro* cell cultures and *in vivo* models. Besides the existence of all these different models, the heterogeneity of MS is very difficult to mimic and there is still no perfect model of the disease (Murta and Ferrari 2013).

3.1. *Ex Vivo* models

Unlike *in vivo* models, organotypic slice cultures represent a less expensive model with lower number of ethical issues, being one of the best alternatives to use on CNS research (Gähwiler 1988). As mentioned above, these models have been used for decades mainly due to their retention of all brain cell types in a 3D tissue environment that replicates *in vivo* conditions that otherwise would not be possible (Gähwiler 1984, Madill et al. 2016). Slice cultures can be prepared from several brain regions, such as the spinal cord and the brain stem, but the most common used in MS research is the cerebellum that, in comparison with other regions, has more abundant white matter with very specific tracts (Mi et al. 2009, Zhang et al. 2011).

Allt et al. (1988) mimicked the demyelination and inflammatory processes associated with MS when exposed organotypic cerebellar slice cultures (OCSC) to the toxin LPC. Demyelination capacity of this toxic detergent was tested by Hall and Gregson (1971) and their results has associate LPC with a rapid demyelination of myelin sheath. It is thought that LPC-induced demyelination recruits the presence of macrophages and microglia to phagocyte myelin residues near demyelinating sites. More recently in 2004, it was reported that LPC-induced demyelination of rat cerebellar slices was followed by the reappearance of myelin sheaths, suggesting that it is possible also to study the remyelination mechanism in this model after a demyelinating insult (Birgbauer et al. 2004).

LPC-treated OCSC are therefore a good model to study demyelination and remyelination, getting closer to the multicellular complexity as well as the structure and functionality of *in vivo* conditions. When compared with *in vitro* models (i.e. cultures of OL precursor cells, or mixed cultures of OL and dorsal root ganglion neurons (Barateiro and Fernandes 2014), this model offers several advantages as the presence of the other glial cells, microglia, astrocytes, as well as CNS, rather than peripheral nervous system-derived neurons that secrete factors that might promote or prevent myelination, accordingly to the surrounding environment in an intact architecture (Dean et al. 2011).

Although this model preserves glial cells and their role in myelination processes, OCSC lack the systemic immune system interactions that would render the model even more complex, but are so important in MS pathogenesis. Apart from this, we consider that OCSC *ex vivo* model is an attractive proposal for MS pathogenesis study with a great potential on the assay of new therapeutic strategies.

3.2. *In Vivo* models

In vivo experimental models applicable to MS research can be classified into three big groups: 1) autoimmune and inflammatory models, including EAE and those that are virus-induced such as Theiler's murine encephalitis virus-induced demyelinating disease (TMEV-IDD); 2) toxic models of demyelination and remyelination, including the chemical lesions by cuprizone, LPC and ethidium bromide; 3) transgenic models, which offer unique approaches to understand pathogenic mechanisms redundancy in biological systems in a more similar way to the disease.

EAE model is a Th cell-mediated autoimmune disease characterized by T-cell and monocyte infiltration in the CNS associated with local inflammation, demyelination and neurodegeneration and is one of the most widely applied models in MS research (Mathew et al. 2013). This model involves the immunization of a susceptible animal with a myelin protein, the main ones are the PLP, MBP or myelin oligodendrocyte glycoprotein (MOG), that will induce brain inflammation, destruction of myelin and consequently axonal damage (Robinson et al. 2014). With the initial inflammation, occurs a profound infiltration by MHC II

restricted $CD4^+$ T-cells that is followed by microglia activation and macrophage recruitment into the lesions, resulting in an acute demyelination and a consequent progressive hind-limb paralysis (Flügel et al. 2001).

Although EAE is commonly employed as a model for MS and as such has been a powerful tool for studying disease pathogenesis as well as potential therapeutic interventions, it presents some limitations on the reproduction of relapses, that difficult the study of remyelination (Ransohoff 2012). In order to understanding remyelination, an important hallmark of RRMS, there was a need to develop other models.

Toxin-induced MS models are among the most significant tools for translational research of defense against progressive disease since enhancing remyelination is deemed crucial for neuroprotection (Ransohoff 2012). The most commonly used toxins for focal demyelination are LPC and cuprizone. Both agents have been used to induce demyelination in certain brain areas such as spinal cord tissue, corpus callosum and hippocampal formation. Similar to *ex vivo* model, LPC is injected into white matter and induces focal plaques of demyelination due to the direct action of the toxin. The destruction phase of myelin shield is quick and is followed by remyelination. On the other hand, the copper chelator cuprizone mixed with chow is fed to vulnerable strains mice at 6 to 9 weeks for 4-6 weeks. Pathologically, cuprizone causes dysfunction of mitochondrial complex IV and is selectively toxic to mature OL (Matsushima and Morell 2001). After 5–6 weeks of cuprizone treatment, the corpus callosum and hippocampus are almost completely demyelinated. Once cuprizone is discontinued and mice are fed normally, demyelination is followed by spontaneous remyelination during subsequent weeks (Skripuletz et al. 2011).

The strength of booth of these directly toxin models lies in the possibility of studying isolated demyelination and remyelination as discrete events with spatio-temporal predictability, providing insights into the cellular and molecular mechanisms of remyelination as well as OL cell death (Miller and Fyffe-Maricich 2010). However, all this dynamic complexity also brings challenges for interpreting all the responses from OPC and other cellular mediators involved in remyelination (Liu et al. 2010). Their main weaknesses and the reason why we do not use these models on this work lies in the absence of ongoing immune

activity, contrary to what is seen in MS. However, it is promising to use these models to identify strategies to enhance remyelination.

Lastly, the generation of transgenic mice has greatly aided to understand pathogenic mechanisms operating in MS. This model is used to assess the role of autoimmune responses to myelin, as well as other mechanisms related to neuronal and OL damage. Transgenic mice have allowed to dissect several human elements involved in MS, by expressing human leukocyte antigens (HLA) haplotypes that are linked to susceptibility to MS disease, leading to a better understanding of the complexity of the disease (Kipp et al. 2012).

In the present work, we decided to use on a first phase the *ex vivo* OCSC model, since it replicates very well the multicellular complexity as well as the structure and functionality of *in vivo* conditions, to assay the efficacy of pentamidine, that targets S100B, in preventing LPC-induced demyelination and inflammation. Since the final goal of our recsearch is to test this drug on a MS *in vivo* model, we next characterized whether the inflammatory S100B-RAGE axis, our target, was also altered in the EAE model. We decide that EAE was the best model to perform this experiment, since it mimics an auto-immune response via T-cell and monocyte infiltration that is followed by microglia activation and macrophage recruitment into the lesions leading to demyelination into an inflammatory milieu.

4. Aims

The major aim of the present study is **to understand if by targeting the inflammatory molecule S100B we may prevent MS-associated pathogenesis**. To address this issue, we first assayed a new S100B-targeting drug in our demyelinating *ex vivo* model, and next characterized the S100B-RAGE axis in a MS *in vivo* inflammatory model, the EAE. Specific airs are:

1) to evaluate whether the direct binding of the drug pentamidine to S100B may prevent the pathogenesis observed in an *ex vivo* demyelination model. For this, we will use OCSC from wild-type (WT) CD1 mice and induce a demyelination insult using LPC. We will keep OCSC for 3 days in culture, at which demyelination will be induced. In parallel, other OCSC will be treated with LPC in the presence of pentamidine. OCSC will then be maintained in culture until 48 hours after the insult. At this point, we will assess demyelination and inflammatory events.

2) to characterize the S100B-RAGE axis in the inflammatory EAE model. For this we will use, in collaboration with JJ Cerqueira, ICVS/i3B's, Universidade do Minho, a cohort of animals that were divided in 3 groups: non-induced, EAE-induced, and EAE-induced and treated at clinical score peak with DMF, a drug in clinical use for MS. We will assay the expression of S100B and RAGE, as well as demyelination and inflammatory markers.

I. Introduction

II. Material and Methods

1. Animals

For the *ex vivo* studies, we used CD1 mice, acquired from Instituto de Higiene e Medicina Tropical (IHMT, Lisboa, Portugal). Animals were supplied with standard laboratory chow and water *ad libitum*.

For the EAE studies, we used Female C57BL/6 wild-type mice that were previously induced to develop EAE with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55). Brain tissue of these mice was obtained in collaboration with J J Cerqueira from Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal.

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. The best efforts were made to minimize the number of animals used and their suffering.

2. Organotypic Cerebellar Slice Cultures (OCSC)

Cerebellar parasagittal slices were obtained from postnatal day 7 (P7) CD1 mouse. Briefly, mice were decapitated and the cerebella were isolated from brains in phosphate buffered saline (PBS), then 400 μm sagittal slices were obtained using a McIlwain tissue chopper. Four slices of different animals were transferred into a membrane culture inserts with 0.4μm pores (BD Falcon,

#353493, Lincoln Park, NJ, USA) in a 6-well cell culture plates that contain 1 mL of medium per well and kept at 37°C, in 5% CO2 conditioned atmosphere. The culture medium consisted of 50% minimal essential media (MEM), (Gibco, Life Technologies, Inc., Grand Islands, USA), 25% of both heat-inactivated horse serum (Gibco) and Earle's balanced salt solution (EBSS, Gibco), 6.5 mg/mL glucose, 25mM HEPES (Biochrom AG, Berlin, Germany), 1% of L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 1% of antibiotic-antimycotic (Sigma-Aldrich). Half media was replaced every day during 3 days in vitro (DIV) before treatment, to allow the clearance of debris and full myelination (Birgbauer et al. 2004). After 3 DIV, culture media was completely removed and replaced by a serum-free media consisting of 98% Neurobasal-A (Gibco) and 2% B-27 (Gibco), supplemented with 1% L-glutamine, 28 mM glucose, 1% of antibiotic-antimycotic and 25 mM HEPES, to improve neuronal viability. Slices were then treated during 18h, at 37°C with lysophosphatidylcholine (LPC) to induce demyelination and in parallel two concentrations of pentamidine were assayed to determine which dose had greater effect without toxicity. So we had 6 treatment groups: (1) Control; (2) 0.5 µg/mL pentamidine (Penta 0.5); (3) 5 µg/mL pentamidine (Penta 5); (4) 0.5 mg/mL LPC; (5) 0.5 µg/mL pentamidine plus 0.5 mg/mL LPC (LPC+Penta 0.5); (6) 5 µg/ml pentamidine plus 5 mg/mL LPC (LPC+Penta 5). After incubation, the LPC-containing medium was removed and the slices were maintained in the absence (Control) or presence of pentamidine in fresh medium during 30h (Figure II.1). Following this recovery period, slices were: (i) stored in

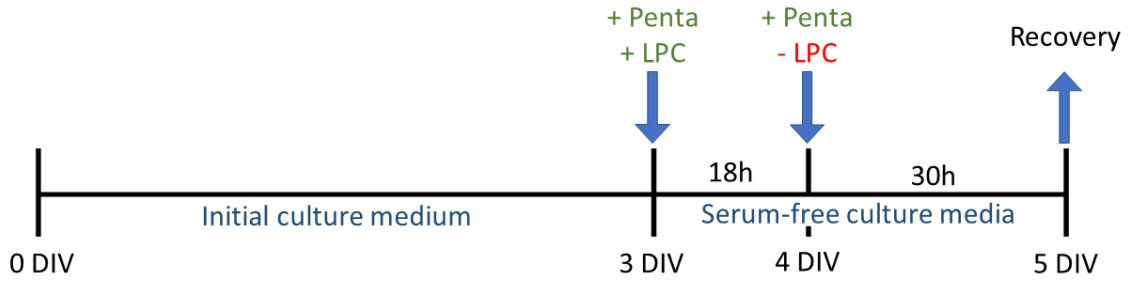

Figure II. 1– Schematic representation of culture treatment. Four slices of different animals were transferred into a membrane culture in a 6-well cell culture plates that contain 1 mL of medium. Half media was replaced every day and was completely removed and replaced by a serum-free media after 3 days in vitro (DIV). Slices were then treated during 18h, at 37°C with lysophosphatidylcholine (LPC, 0.5 mg/mL) to induce demyelination and two concentrations of pentamidine (0.5 or 5 µg /mL). After incubation, the LPC-containing medium was removed and the slices were maintained in the absence (Control) or presence of pentamidine in fresh medium during 30h.

TRIzol® reagent, at -20°C, for RNA extraction; or (ii) fixed in 4% paraformaldehyde (PFA) for immunohistochemistry assays.

3. Experimental autoimmune encephalomyelitis (EAE)

Disease induction was performed at 9-11 weeks of age, using a commercial kit (EK-2110; Hooke Laboratories, Lawrence, MA, USA), according to the manufacturer's instructions. Animals were immunized subcutaneously with 200 µg of myelin oligodendrocyte glycoprotein 35-55 (MOG35-55), emulsified in complete Freund's adjuvant (CFA), at the upper and lower back (100µL of emulsion per site of injection). Pertussis toxin (PTX) in PBS was administered intraperitoneal after 2 and 24 hours of immunization (227 ng of PTX per injection). Non-induced age-matched female littermates were used as controls. Non-induced animals were injected subcutaneously with an emulsion of PBS in CFA (Difco Laboratories, Detroit, USA), and were injected with PTX at the same concentration and time-points as the EAE animals.

Animals were daily weighted and monitored for clinical symptoms of disease, and the evaluation of the clinical disease score was performed with the experimenter blinded to the treatment group. At the clinical peak, from day 18 until day 36 post-EAE induction, animals were treated with 15 µg/g of DMF (Sigma-Aldrich) in 0.8% hypromellose by oral gavage, two times per day, accordingly to Linker and colleagues (2011). Non-induced animals were treated with vehicle solution and followed the same treatment timeline. Figure II.2 illustrate the timeline of the experimental procedure.

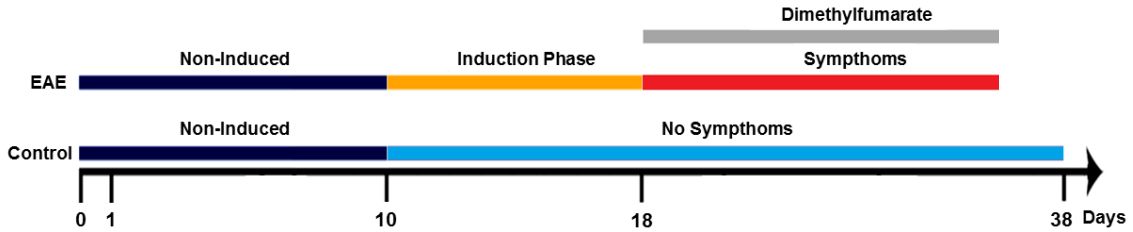

Figure II. 2 – Schematic representation of the experimental procedure. Disease induction was performed at 9-11 weeks of age with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) at the day 10. Non-induced animals were injected subcutaneously with an emulsion of PBS in CFA. From day 18 until day 36 post-EAE induction, animals were treated with DMF and non-induced animals were treated with a vehicle solution. Both groups were sacrificed at day 38.

Disease severity was assessed as previously described (Stromnes and Goverman 2006), with few changes, as follows: 0 – no clinical symptoms;

0.5 – partially limp tail; 1 – paralyzed tail; 1.5 – at least one hind limb falls through consistently when the animal is placed on a wire rack; 2 – loss in coordinated movement, wobbly walk; 2.5 – dragging of hind limbs; 3 – paralysis of both hind limbs; 3.5 – hind limbs paralyzed and weakness of forelimbs; 4 – complete hind limbs paralysis and partial forelimbs paralysis; 4.5 – animal is not alert, no movement; 5 – moribund state or death.

Non-induced, vehicle- and DMF-treated EAE animals were sacrificed at the light phase of the diurnal cycle, at day 38. Animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (150 mg/kg, Imalgene® 1000) plus medetomidine hydrochloride (0.3 mg/kg, Dorben®).

For histological analysis, mice were transcranial perfused with cold 0.9% saline solution, followed by perfusion with 4% PFA. The brains were then collected, left in 4% PFA for 24 hours, and switched to a 30% sucrose solution for 24 hours. Then the brains were embedded in Tissue-Tek® O.C.T.™ compound (Sakura Finetek, Japan), snap-frozen and sent to our laboratory.

4. Total RNA Extraction, and Semi-quantitative RealTime-PCR

In order to determine the expression levels of diverse genes of interest, total cytoplasmic RNA was isolated from OCSC 5 DIV treated slices and EAE slides using the TRIzol® reagent method in accordance with the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA) and RNA concentration was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 200 ng of total RNA were reversely transcribed using the SensiFAST cDNA Synthesis Kit (Bioline), under manufacturer's instructions. Quantitative RealTime-PCR (qRealTime-PCR) was performed on a real-time PCR detection system (QuantStudio 7 Flex Real-Time PCR System, Applied Biosystem, Madrid, Spain) using a SensiFAST SYBR® Hi-Rox Kit (Bioline) under optimized conditions: 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s and 62ºC for 1min. The PCR was performed in 384-well plates with each sample performed in duplicate. The sequences used as primers are listed in the Table II. 1. Relative mRNA concentrations were calculated using the Pfaffl modification of the ΔΔCT equation, where CT is the cycle number at which fluorescence passes the threshold level of detection, taking into account the efficiencies of individual genes. The results were

normalized to the housekeeping gene β-actin in the same sample and the initial amount of the template of each trial was determined as relative expression by the formula $2^{-\Delta\Delta CT}$. ΔCT is the value obtained for each sample by performing the difference between the mean CT value of each gene of interest and the mean CT value of β-actin. ΔΔCT of one sample is the difference between its ΔCT value and the ΔCT of the sample chosen as control.

Table II. 1– List of pairs of primers used for qRealTime-PCR assays.

Gene	Forward	Reverse
B-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt
Cd11b	cagatcaacaatgtgaccgtatggg	catcatgtccttgtaactgccgcttg
HMGB1	ctcagagaggtggaagaccatgt	gggatgtaggttttcatttctctttc
IL-1β	caggctccgagatgaacaac	ggtggagagctttcagctcata
IL-10	atgctgcctgctcttactga	gcagctctaggagcatgtgg
MBP	ccatccaagaagaccccaca	cccctgtcaccgctaaagaa
NG2	gggctgtgctgtctgttga	tgattcccttcagctaaggca
PSD-95	cgaggatgccgtggcagcc	catggctgtggggtagtcagtgcc
RAGE	ttcacgacgaagttccaaacaggt	gttctaggaggactggggtg
Syn	tcaggactcaacacctcagtgg	aacacgaaccataagttgccaa
S100B	tgtagaccctaacccggagg	tgcatggatgaggaaggcat
TLR4	acctggctggtttacacgtc	gtgccagagacattgcagaa
TNF-α	tactgaacttcggggtgattggtcc	cagccttgtcccttgaagagaacc

5. Immunostaining procedure

For immunostaining procedure, insert membranes, which contain the fixed slices, were cut, placed into a cover glass and blocked with 1nM HEPES, 2% heat-inactivated horse serum,10% heat-inactivated goat serum (Biochrom), 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.25% Triton X-100 (Roche Diagnostics, Indianapolis, USA) in Hank's balanced salt solution (HBSS, Gibco) for three hours, at room temperature. The same procedure was done for EAE slides except that blocking was only performed for one hour. After blocking, slices/slides were incubated with primary antibody (Table II. 4), diluted in the blocking solution, for 24h, at 4°C. Following this, slices/slides were washed three

times for 15 min each with PBS with 0.2% Triton X100® (PBS-T) and incubated with secondary antibodies (Table II. 5) in blocking solution for another 24 h at 4ºC. Slices/slides were then washed three times for 15 min each with PBS-T, incubated with DAPI (1:1000, 3 min) to stain the nuclei, washed three times for 15 min each with PBS-T and mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) for fluorescence microscopy. Fluorescent images were acquired using Leica DMi8-CS inverted microscope with Leica LAS X software (version 3.1.1.) and analyzed with ImageJ software.

Table II. 2– List of primary antibodies used for immunoshistochemistry assays.

Antibody	Host	Brand	Reference Number	Dilution
GFAP	Mouse	Novocastra	6035278	1:100
Iba-1	Babbit	Wako	079-19741	1:250
NG2	Rabbit	Temecula	AB5320	1:200
MBP	Rat	BioRad	MCA409S	1:200
NF-160	Mouse	Novocasta	NCL-NF160	1:200
RAGE	Rabbit	Abcam	Ab3611	1:100
S100B	Rabbit	Abcam	ab52642	1:200

Table II. 3– List of secondary antibodies used for immunoshistochemistry assays.

Antibody	G	Brand	Reference Number	Dilution
Alexa 488 anti-mouse	Goat	Invitrogen	A10680	1:1000
Alexa 488 anti-rabbit	Goat	Invitrogen	A11008	1:1000
Alexa 594 anti-rat	Donkey	Invitrogen	A21209	1:1000
Alexa 594 anti-mouse	Goat	Invitrogen	A11005	1:1000

The number of positive cells for each antibody was counted from 8 bit.lsm files of 1390x1036 pixel resolution images. Approximately, 4-5 images were captured per slice/slide per condition, thus reducing any variations in image acquisition. Then the total cell number of MBP, NG2 and Iba-1 was counted for each separated stack of every acquired image using ImageJ software. Regarding myelination, percentage of the area immunoreactive of MBP and NF-160 was measured and percentage of myelinated fibers was obtained by the ratio between

the area of co-localization of NF-160 and MBP and the total area occupied by NF-160.

6. Histopathological analysis

Using a cryostat (LEICA CM 3050S) 20 µm coronal slides were obtained from the brains received from ICVS/i3B's, Universidade do Minho. Slides were stained with luxol fast blue solution at 56 ºC overnight, excess stain was rinsed off with 70% ethyl alcohol and washed with distilled water for 5 minutes. Differentiation of the slides was done in lithium carbonate solution for 5 minutes and rinsed in distilled water for another 5 minutes. Then slides were stained with hematoxylin for 10 minutes and washed with tap water for 5 minutes, hydrochloric acid was then used for 5 seconds to differentiate and rinsed with tap water one last time for 5 minutes. Finally, slides were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) for optical microscope. Images were acquired using a Leica DC 100 camera (Leica, Wetzlar, Germany) adapted to an Axioskop microscope (Zeiss). Images were then merged using Adobe Photoshop CC 2017 software. Three specific regions usually related to demyelination were used to assess the degrees of demyelination in a blinded manner for each mouse (Figure II. 3). To quantify the level of demyelination we followed the standards described by Han et al. (2013): 0 - Normal white matter; 1 - Rare foci; 2 - A few areas of demyelination; 3 - Confluent perivascular demyelination; 4 - Massive demyelination involving one half of the brain; and 5 - Extensive demyelination involving the whole brain.

7. Statistical Analysis

All results are presented as mean ± SEM. Differences between two groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance or by one-way ANOVA with Tukey post-test for multiple comparisons, using GraphPad PRISM 5.0 (GraphPad Software, San Diego, CA, USA), as appropriate. The P-values of $P<0.05$ and $P<0.01$ were considered as being statistically significant.

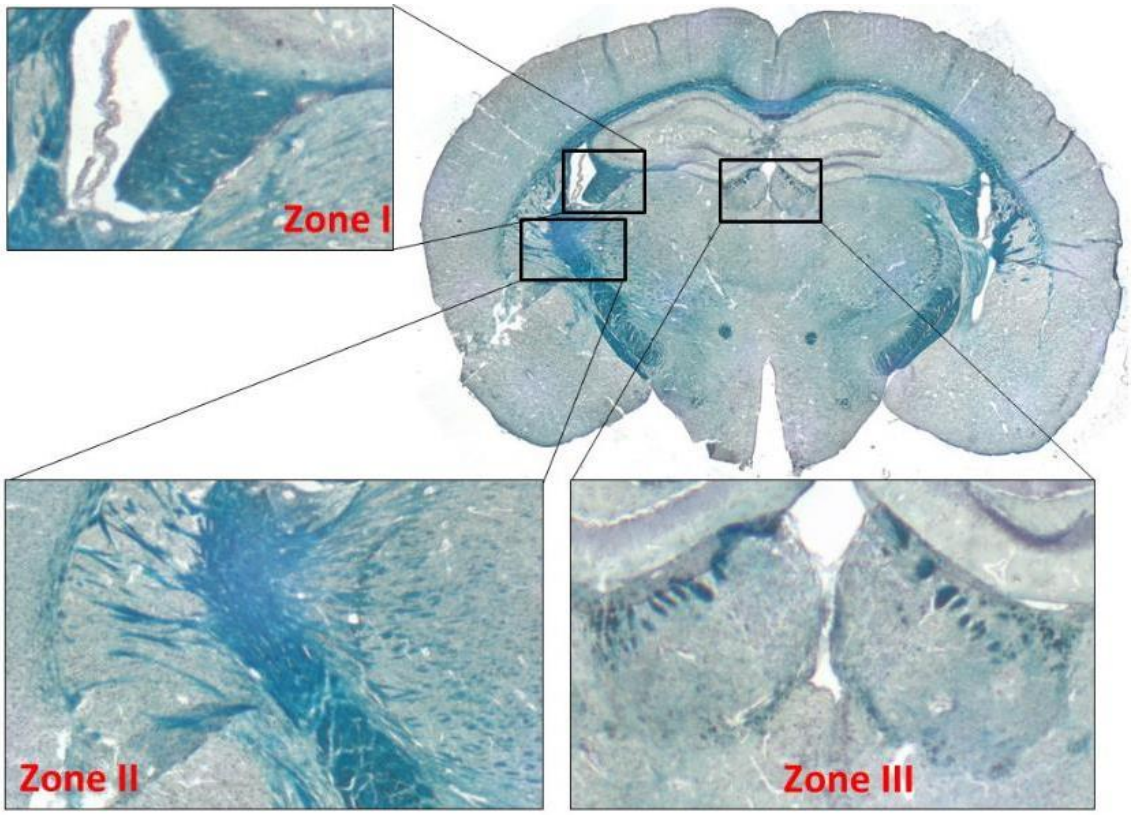

Figure II. 3 – Representative brain coronal slices (20 µm) of the EAE-induced model indicating the evaluated regions: (I) fimbria, (II) internal capsule and (III) perivascular zone.

III. Results

1. Induced demyelination of organotypic cerebellar slice cultures increases S100B expression

Abnormal levels of S100B have been detected in MS patients in both CSF and post-mortem plaques (Bartosik-Psujek et al. 2011) and more recently, our group confirmed a significant increase of S100B production in serum at the time of diagnosis in RRMS patients (Barateiro et al. 2016). To confirm that S100B was being expressed in our *ex vivo* model of demyelination, as usual in our lab (Barateiro et al. 2016), we decided to evaluate the expression of this molecule extracted from OCSC 48h post-incubation with the demyelinating agent LPC. As illustrated in Figure III. 1A, LPC insult markedly upregulated S100B protein expression that partially co-localized with GFAP-positive astrocytes. Corroborating these results LPC incubation also promoted a significant increase in S100B gene expression (1.96-fold, $p<0.05$).

These results validate our *ex vivo* model of demyelination for the expression and production of S100B.

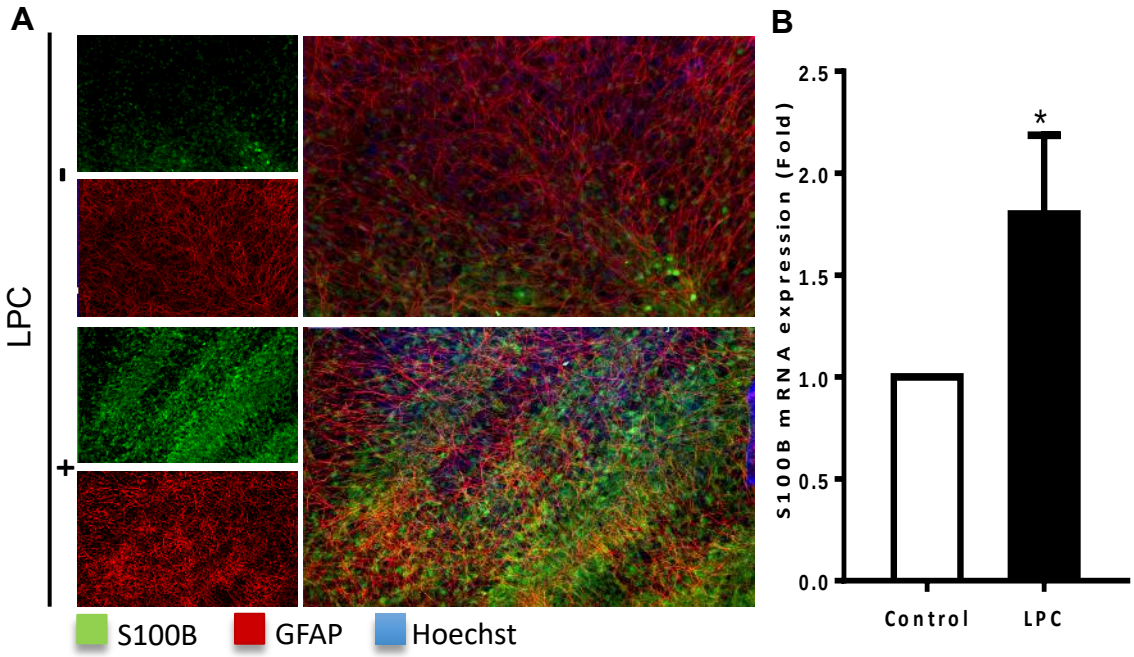

Figure III. 1 – A demyelinating insult induces S100B expression in organotypic cerebellar slice cultures. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC) at 3 days in vitro (DIV) for 18 hours. At 48 hours following the incubation with LPC, OCSC were immunostained for S100B (green) and astrocytes (GFAP) (red), and stained with Hoechst to detect nuclei (blue). **(A)** Representative images of immunohistochemistry for S100B and GFAP (Magnification: 40x). **(B)** Relative levels of the gene expression of S100B were also determined 48 hours post LPC treatment by qRealTime-PCR. Results are mean ± SEM. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate*P<0.05 vs. Control.

2. Demyelination-induced expression of S100B is attenuated in the presence of Pentamidine

Knowing that S100B is being overly expressed in response to LPC-induced demyelination, we decided to evaluate the effect of pentamidine, an S100B inhibitor, using two different concentrations (0.5 µg/mL and 5 µg/mL) as previously reported to reduce inflammation in an Alzheimer's disease model (Cirillo et al. 2015). As illustrated in Figure III. 2A, high S100B gene expression promoted by LPC insult is completely abrogated by both 0.5 µg/mL and 5 µg/mL pentamidine to control levels ($p<0.01$). Interestingly, although the low concentration of pentamidine did not appear to affect the gene expression of S100B in the absence of LPC, the high concentration partially increased it.

Next, we decided to evaluate the gene expression of S100B specific receptors, the RAGE and TLR4. As illustrated in Figure III. 2B, upon LPC insult, in parallel to increased expression of S100B, RAGE gene expression is also increased (3.72-fold, $P<0.05$). Interestingly, co-incubation with 0.5 µg/mL

pentamidine, decreased RAGE gene expression of levels close to the control expression (1.09-fold, $p<0.05$), while co-incubation with 5 µg/mL pentamidine, exhibits a partial but not significant decrease (2.18-fold). Although a similar pattern is observed for TLR4 gene expression, no significant changes were obtained due to a higher variability, possibly suggesting that S100B-RAGE axis is more involved in our experimental condition.

As previously mentioned S100B might stimulate its own expression via RAGE engagement. These data suggest that pentamidine may have a possible role in sequestering extracellular S100B and, in this case, preventing S100B interaction with RAGE receptor resulting in an impaired gene expression of both S100B and RAGE.

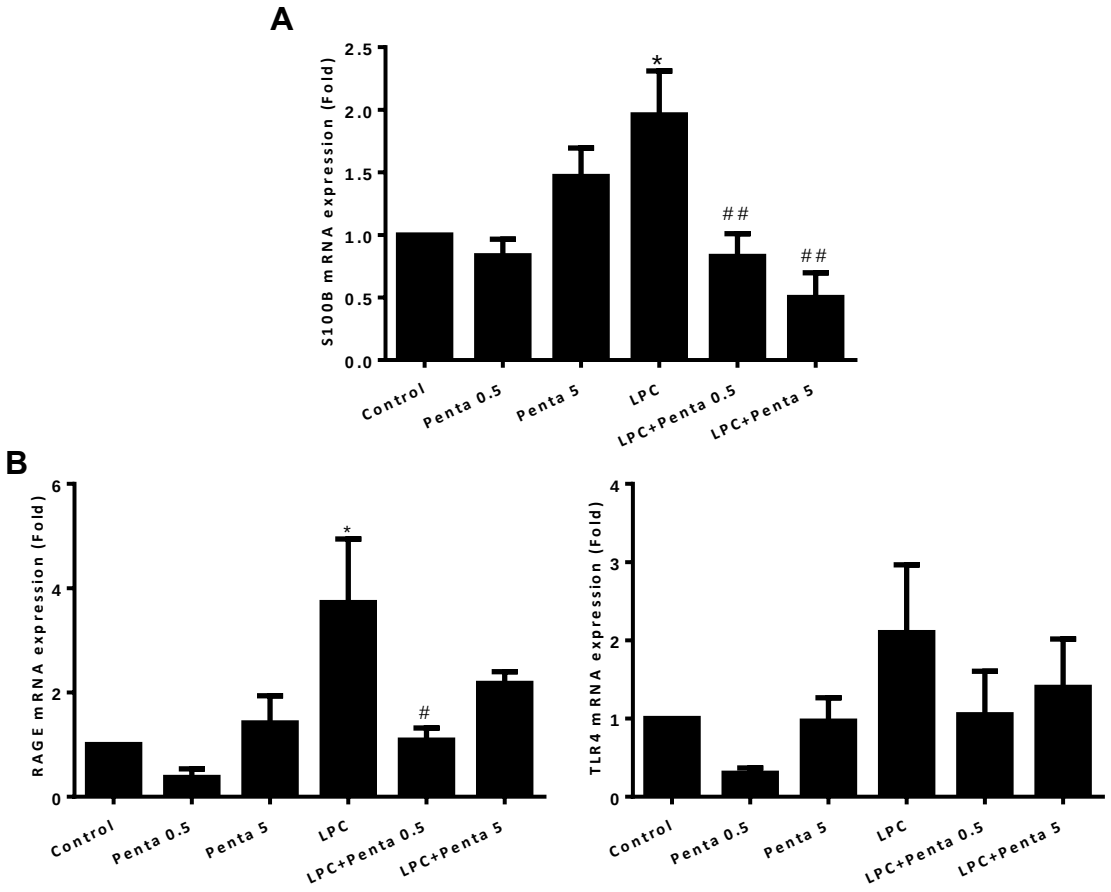

Figure III. 2 – Pentamidine attenuate the expression of S100B and its receptors after LPC insult. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC) at 3 days in vitro (DIV) for 18 hours. Pentamidine treatment (0.5 or 5 µg/mL) occurred during the insult and was maintained during the 30h recovery. Total mRNA was extracted from OCSC at 48h post-LPC and gene expression was analyzed by qRealTime-PCR to detect **(A)** S100B and its specific receptors **(B)** TRL4 and RAGE. Results are mean ± SEM. *P<0.05 vs Control. #P<0.05 and ##P<0.01 vs LPC alone. Magnification of imagens: 40x.

3. Pentamidine partially attenuates demyelination induced by LPC

Knowing that S100B is being overly expressed in response to LPC-induced demyelination, we wanted to find out whether this increase in extracellular S100B could contribute to LPC-induced demyelination related events. For this end, we evaluated the percentage of myelinated fibers and neurofilaments in OCSC at 48h post-LPC insult.

As depicted in the Figure III. 3, LPC stimulus effectively damaged myelin sheaths as expected, which is corroborated by the marked decrease observed in myelinated fibers (0.48-fold $P<0.01$). Interestingly, co-incubation with pentamidine partially prevented the demyelinating insult caused by LPC stimulus as observed in Figure III.3 A, corroborated by an increase in myelinated fibers over LPC values (40.9% and 50.8% for 0.5 µg/mL and 5 µg/mL, $p<0.01$) (Figure III. 3 B).

The decrease in myelinated fibers may be related with the decrease in neurofilaments caused by LPC toxicity. Indeed, as depicted in Figure III. 3C, NF-160 staining shows a marked decrease with LPC insult (0.56-fold% $p<0.01$). This damage was partially prevented when OCSC were co-incubated with 0.5 µg/mL and 5 µg/mL pentamidine (58.7% and 59%, respectively, $p<0.05$).

These results indicate that pentamidine, by acting on excessive released S100B, may have an important role on the prevention of the demyelination related events. To note, 5 µg/mL pentamidine seems to partially damage by itself myelin sheaths and also has a negative effect on neurofilaments (0.72-fold and 0.63-fold, respectively, $p<0.01$). These results may suggest a toxic event of this drug concentration, either directly or via inhibition of the physiological low levels of S100B that have been reported to play a neurotrophic role.

4. Pentamidine attenuate loss of mature oligodendrocytes and increase the number of immature oligodendrocytes following LPC

Demyelinating events in MS are usually followed by spontaneous remyelination, in which new myelin sheaths are elaborated, leading to a total or partial clinical remission of the symptomatology. This remyelination involves the

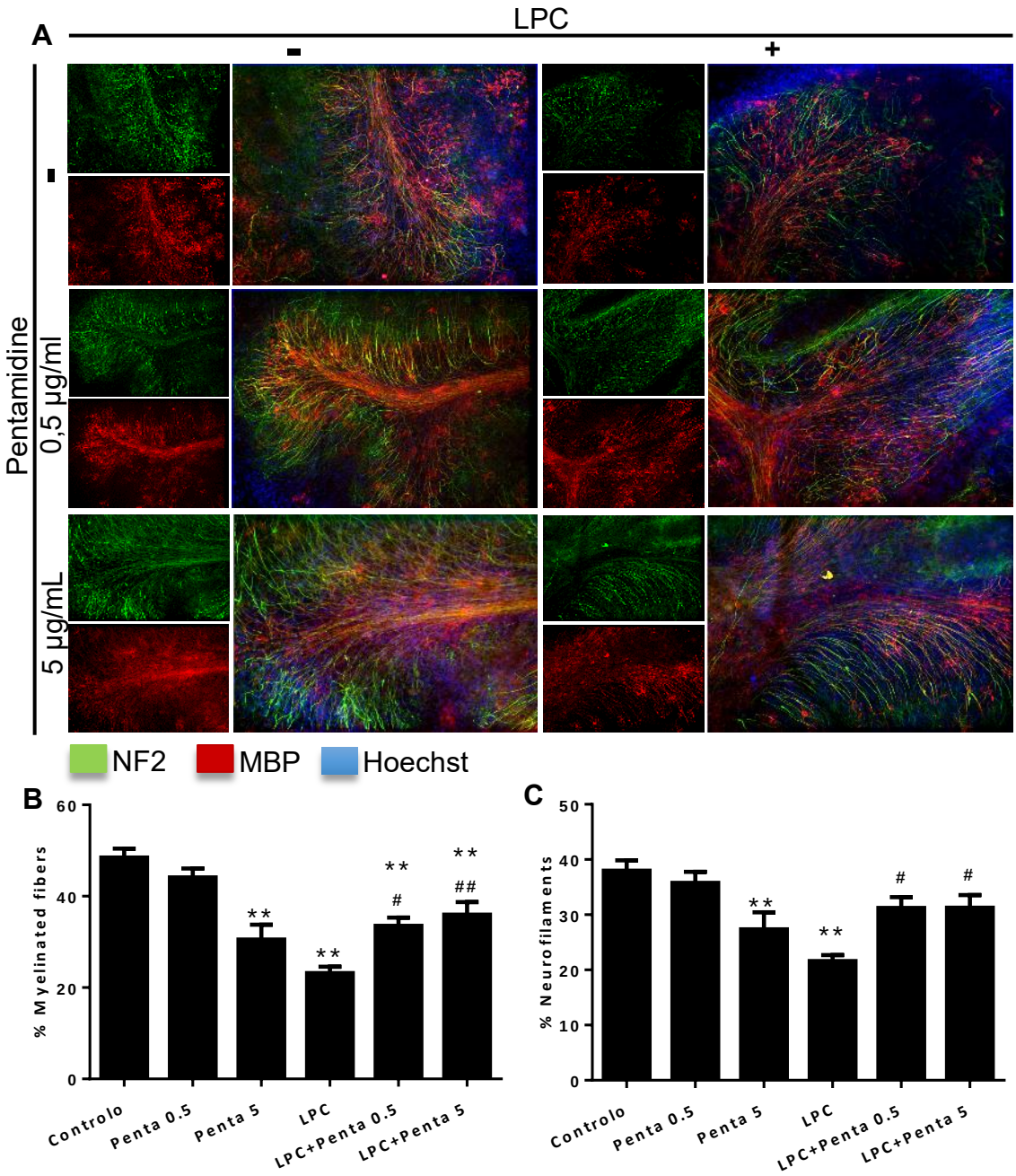

Figure III. 3 – Pentamidine attenuates the demyelination and axonal damage caused by LPC. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC) at 3 days in vitro (DIV) for 18 hours. Pentamidine treatment (0.5 or 5 µg/mL) occurred during the insult and was maintained for the 30h recovery. **(A)** Double immunostainings were performed in fixed OCSC at 48h post-LPC to observe the axons, neurofilaments (NF-160, green) and mature oligodendrocytes, myelin basic protein (MBP, red). **(B)** Quantification of the percentage of myelinated fibers was calculated by the ratio between the area of co-localization of NF-160 and MBP and the total area occupied by NF-160. **(C)** Area occupied with NF-160 staining was obtained by averaging the percentage of NF-160 area of each co-staining. Results are mean ± SEM. **P<0.01 vs Control. #P<0.05 and ##P<0.01 vs LPC alone. Magnification of imagens: 40x.

recruitment and proliferation of endogenous OPC to the injured area (Dubois-Dalcq et al. 2005). Our previous results showed a significant prevention of LPC-induced demyelination by pentamidine. Therefore, we decide to explore whether the values of OL vs OPC could also be altered. As shown in Figure III.4, LPC-

induced demyelination caused a marked decrease in the number of MBP^+ cells (0.32-fold, $p<0.01$) and a consequent increase in the number of $NG2^+$ cells (1.8-fold, $p<0.01$), possibly indicating an attempt to replace the lost cells. Interestingly, co-incubation with pentamidine reverted this panorama by partially increasing the number of MBP^+ cells (2.18-fold and 2.45-fold for 0.5 µg/mL and 5 µg/mL, $p<0.01$) and reducing the $NG2^+$ cells (0.77-fold, $p<0.05$, and 0.86-fold for 0.5 µg/mL and 5 µg/mL) over LPC treatment (Figure III.4B, C). These results shown that $NG2^+$ cells begin to proliferate as soon as the LPC insult to MBP^+ cells occur. In the presence of pentamidine there is a reduction of demyelination, corroborated by increased number of MBP^+ cells and a reduced need for $NG2^+$ OPC proliferation.

5. Pentamidine attenuate inflammatory response triggered by demyelination

In MS, demyelination is characterized by astrocytic and microglial activation involving an exacerbated production of inflammatory cytokines and chemokines (Mosser 2003, Sospedra and Martin 2005).

Taking this into account we intended to evaluate the expression of cytokines that are associated with the inflammatory process. As shown in Figure III. 5, LPC insult promoted a marked increase in the expression of first line cytokines TNF-α and IL-1β (3.52-fold, $p<0.01$ and 3.06-fold, $p<0.05$, respectively), as well as the alarmin HMGB1 (3.21-fold, $p<0.01$). Although we also detected increases in the gene expression of the pleotropic cytokine IL-6 (4.89-fold) and the anti-inflammatory IL-10 (1.9-fold), these differences were not significant over control values. Interestingly when co-incubated with pentamidine, in both concentration (0.5 µg/mL and 5 µg/mL) gene expression of TNF-α, IL-1β and HMGB1 were significantly decreased ($p<0.05$) (Figure III. 5A). These results clearly suggest that pentamidine is able to somehow prevent the inflammatory response. On the other hand, while the gene expression of IL-6 showed a slight non-significant reduction over control values, the mRNA levels of IL-10 remained elevated (Figure III.5B).

Collectively, these results suggest that pentamidine is able to prevent not only the demyelination elicited by LPC insult, but also the exacerbated production of pro-inflammatory factors, although not affecting the anti-inflammatory ones.

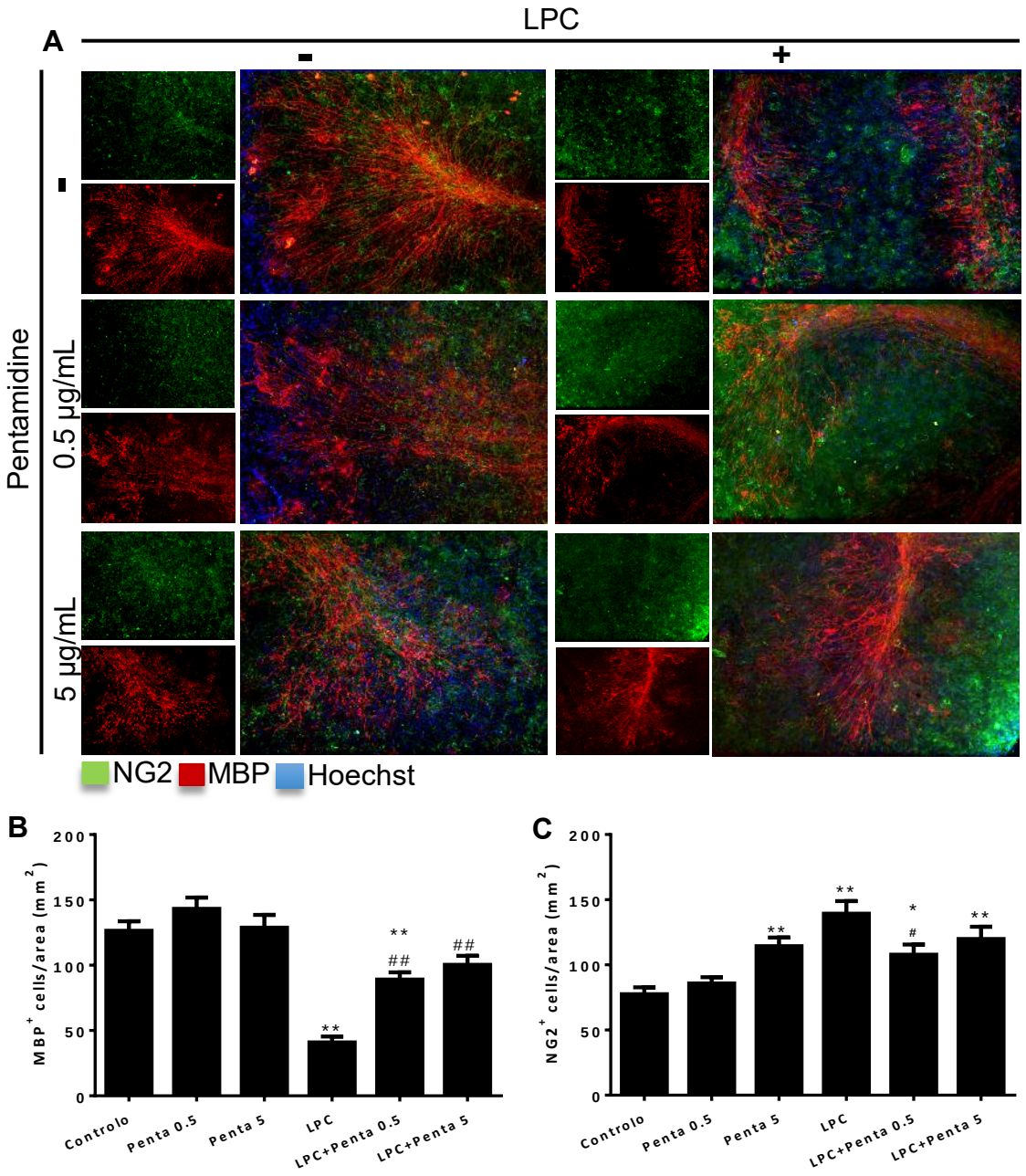

Figure III. 4 – Pentamidine prevents the loss of mature oligodendrocytes and the proliferation of oligodendrocyte precursor cells. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC) at 3 days in vitro (DIV) for 18 hours. Pentamidine treatment (0.5 or 5 µg/mL) occurred during the insult and was maintained for the 30h recovery. **(A)** Double immunostainings were performed in fixed OCSC at 48h post-LPC to observe oligodendrocyte precursor cells (OPC), neural/glial antigen 2 proteoglican (NG2, green) and mature oligodendrocytes, myelin basic protein (MBP, red). MBP+ **(B)** and NG2+ cells **(C)** were quantified for each stack and normalized to the total area of the image. Results are mean ± SEM. *P<0.05 and **P<0.01 vs Control; #P<0.05 and vs LPC alone. Magnification of imagens: 40x.

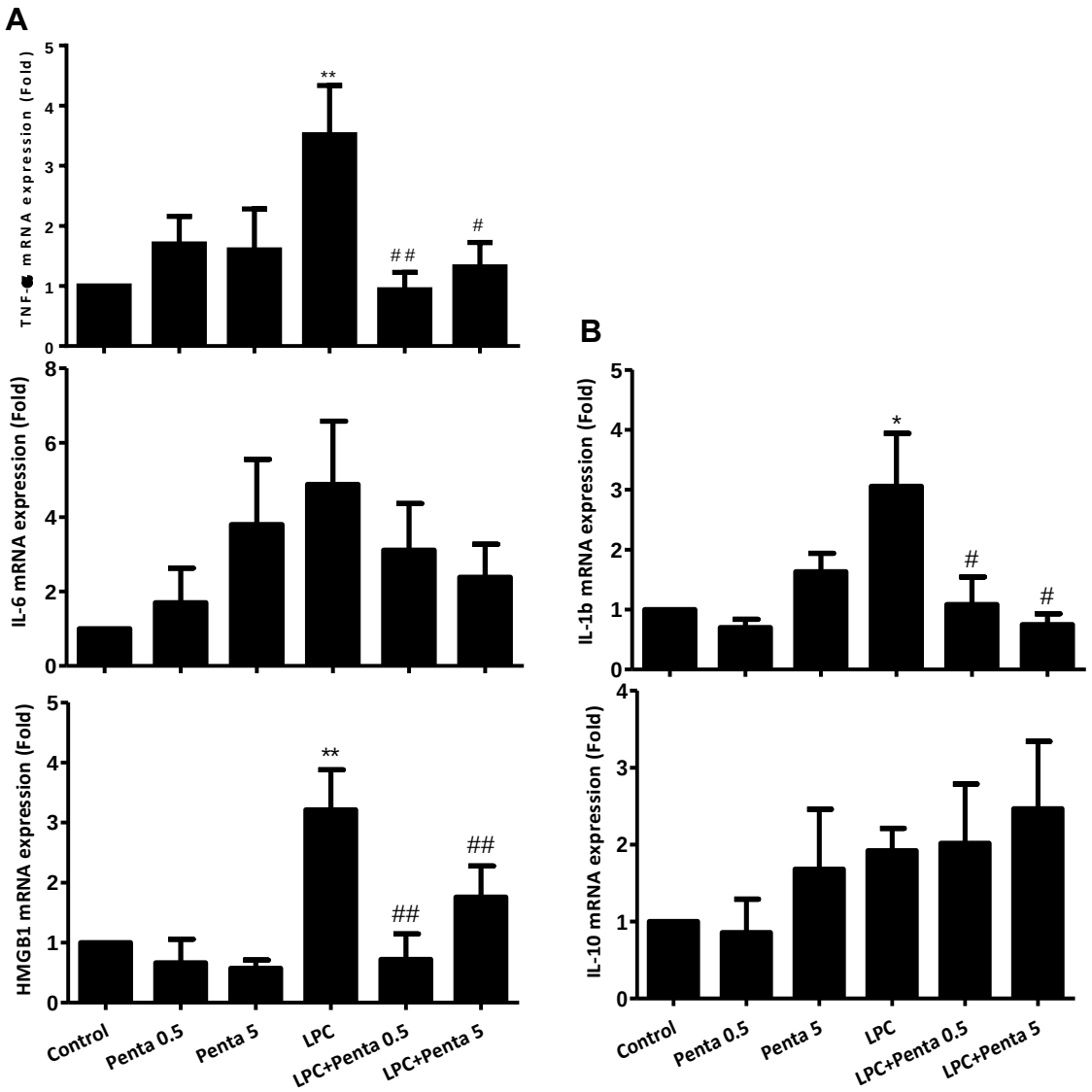

Figure III. 5 – Pentamidine significantly decreases the gene expression of TNF-α, IL-1β and HMGB1 induced by LPC-demyelination. Organotypic cerebellar slice cultures (OCSC) were exposed to lysophosphatidylcholine (LPC) at 3 days in vitro (DIV) for 18 hours. Pentamidine treatment (0.5 or 5 µg/mL) occurred during the insult and was maintained for the 30h of recovery. Total mRNA was extracted from OCSC at 48h post-LPC and gene expression was analyzed by qRealTime-PCR with specific primers to **(A)** TNF-α, IL-1β, HMGB1 and IL-6, as well as to **(B)** IL-6 and IL-10. Results are mean ± SEM. *P<0.05 and **P<0.01 vs Control. #P<0.05 and ##P<0.01 vs LPC alone.

6. EAE induced demyelination is prevented upon DMF treatment

In order to assay pentamidine as a potential therapeutic strategy on an *in vivo* model of MS, we must first be sure that the S100B/RAGE axis is highly expressed in the chosen model. To address this issue, we decided to characterize the S100B-RAGE axis in the inflammatory EAE model. For this, we used, in collaboration with JJ Cerqueira, ICVS/i3B's, Universidade do Minho, a cohort of animals that were divided in 3 groups: non-induced, EAE-induced, and

EAE-induced plus DMF treatment initiated at peak clinical score as determined in JJ Cerqueira lab (Figure III.6).

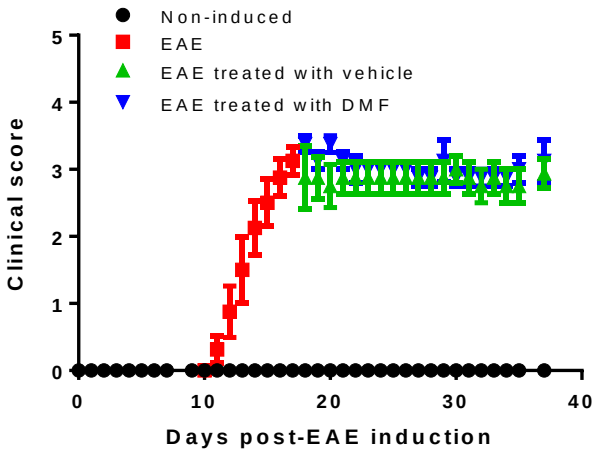

Figure III. 6 – Clinical course of the EAE model. Clinical score is used to establish a numeric value to the disease severity. The severity was assessed as follows: 0 – no clinical symptoms; 0.5 – partially limp tail; 1 – paralyzed tail; 2 – loss in coordinated movement, wobbly walk; 3 – paralysis of both hind limbs; 4 – complete hind limbs paralysis and partial forelimbs paralysis; 5 – moribund state or death.

Although no differences were observed for the clinical score upon DMF treatment, some benefits were reported by JJ Cerqueira lab in terms of learning ability. Thus, we decided to evaluate how myelination could be impaired in the EAE group and whether DMF treatment during the chronic phase could be ameliorating it. Brain slides were stained with luxol fast blue solution and three specific regions, usually related to demyelination [e.g. (I) fimbria (II) internal capsule and (III) perivascular zone], were used to assess the degrees of demyelination for each mouse. As illustrated in Figure III. 7A, B, EAE-induced mouse effectively show damaged myelin sheaths as expected (4.8-fold, $p<0.01$) that was reduced upon DMF treatment by 70% ($p<0.05$).

These results were supported by gene expression analysis of immature (NG2) and mature (MBP) OL markers. As shown in Figure III. 7 C, we observed that EAE markedly decrease both NG2 and MBP gene expression (0.038-fold and 0.18-fold, respectively, $p<0.01$) over control values, suggesting a loss of OL that is no longer compensated by OPC, consistent with a chronic and long-lasting demyelination. On the other hand, when we looked to specific synaptic markers, we observed that EAE also reduced the gene expression of PSD-95 and synapthophysin (0.38-fold, $p<0.05$ and 0.45-fold, $p<0.01$ respectively), indicating neuronal dysfunction. Interestingly, animals treated with DMF, showed an

increase of both immature OPC (~88.7%, p<0.01), and mature OL (~31.3%, p<0.05) markers, corroborating once again the observed results for myelination. Relative to the expression of synaptic markers, animals treated with DMF showed a marked increase (>100% for PSD-95, p<0.05) and (~87% for synapthophysin, p<0.01). Our results confirm, as expected, that the EAE chronic model elicit in

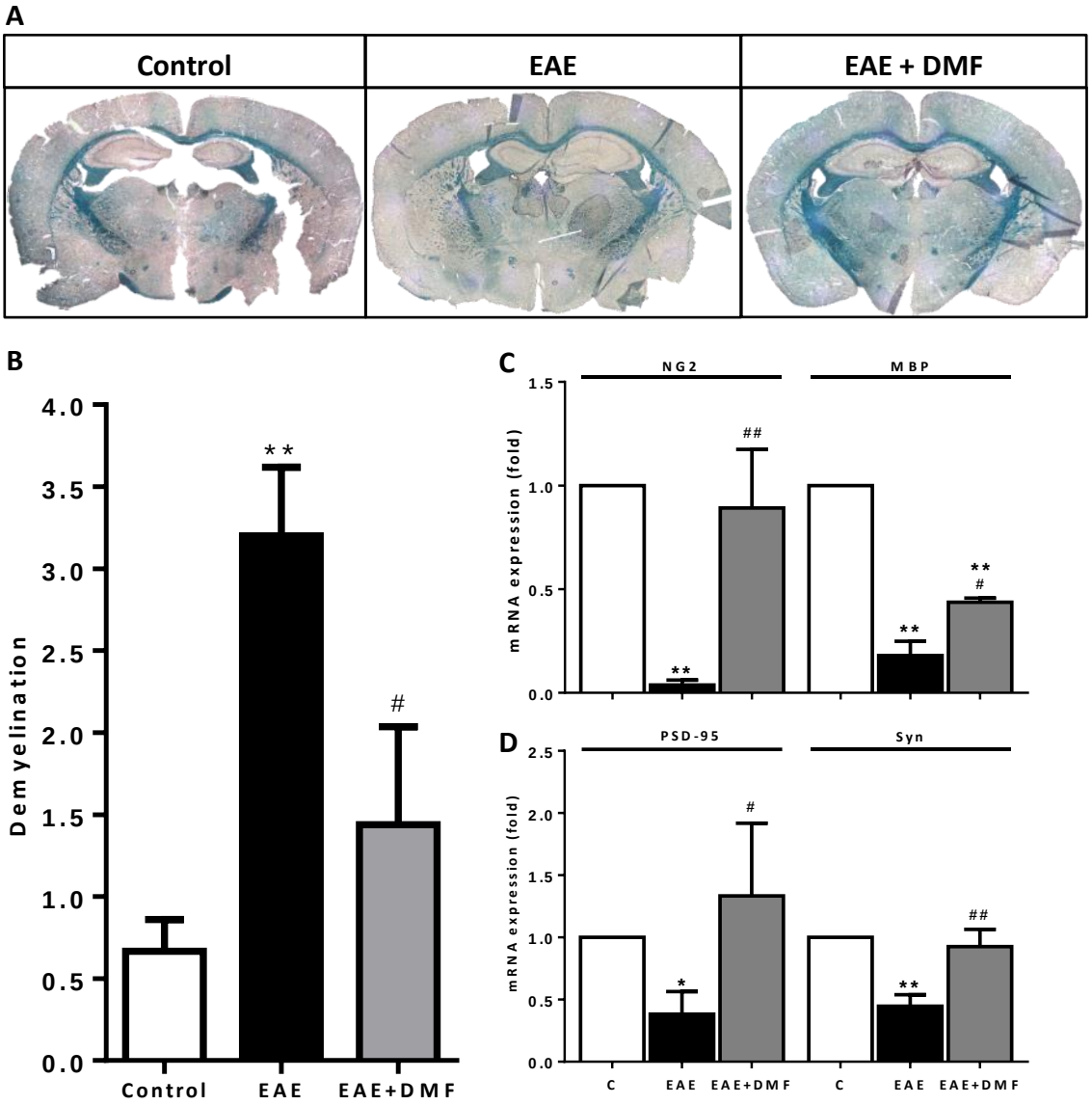

Figure III. 7 – DMF treatment ameliorates brain histopathology lesions and expression of OL and synaptic markers. (A) Representative images of brain lesions of EAE treated mouse with DMF during the chronic phase of the disease (day 38) (n=4). Luxol fast blue (myelin) staining shows a great loss of myelin in EAE group that was prevented upon DMF treatment. Bregma: -1,64. **(B)** Graph bars represent a score of demyelination based on slides observation (0 - no demyelination; 5 - complete demyelination). **(C)** Relative levels of immature (NG2) and mature (MBP) OL markers were determined by qRealTime-PCR. **(D)** Relative levels of synaptic markers (PSD-95 and synaptophysin) were determined by qRealTime-PCR. Results are mean ± SEM. *p<0.05, **P<0.01 vs. Control and #P<0.05, ##P<0.01 vs EAE.

parallel with demyelination, an oligodendrogenesis failure accompanied by loss of synaptic markers that is partially reverted with DMF treatment.

7. EAE induced astrogliosis and increased S100B expression were prevented by DMF treatment

As previously mentioned S100B is mostly expressed by astrocytes and act as a signaling molecule through engagement of the RAGE receptor. S100B exerts beneficial or detrimental effects in a concentration-dependent manner. Knowing that induced-EAE presented a great loss of myelin that was reduced after DMF treatment, we decided to evaluate if this loss is accompanied by an increased expression of S100B, namely with an astrocyte origin.

For this end, induced-EAE slides were double immunostained for S100B and astrocytes (GFAP) and $S100B^+$ and $GFAP^+$ cells were quantified for each stack in the three most affected regions: (I) fimbria (II) internal capsule and (III) perivascular zone, and normalized to the total area of the image. As shown in Figure III. 8, EAE insult caused a marked overall increase of S100B and GFAP ($p< 0.01$) in all the regions studied that was still maintained at the 38-days endpoint. Interestingly, both S100B expression and astrogliosis decreased after DMF treatment by more than 85% ($p<0.01$), suggesting a reduction of the S100B-related inflammatory response. Next, we evaluated the density of astrocytes expressing S100B by co-localization and we observed a significant higher number of $S100B^+/GFAP^+$ cells in EAE animals, namely in region II (5.51-fold, $p<0.01$) and III (3.17-fold, $p<0.01$) over control values, that was once again prevented by DMF treatment (~87% and >100%, respectively, $p<0.01$).

8. EAE induces increased RAGE expression which is prevented following DMF treatment

S100B act as a signaling molecule through engagement specific receptors and our previous results suggest that S100B-RAGE signaling must be the most relevant in demyelinating conditions. So, knowing that induced-EAE caused a marked overall increase of S100B, we decided to evaluate if this increment was accompanied by an increased expression of RAGE receptor. For this end, brain slides were immunostained for RAGE and $RAGE^+$ cells were quantified for each stack in the same three regions and normalized to the total area of the image. As

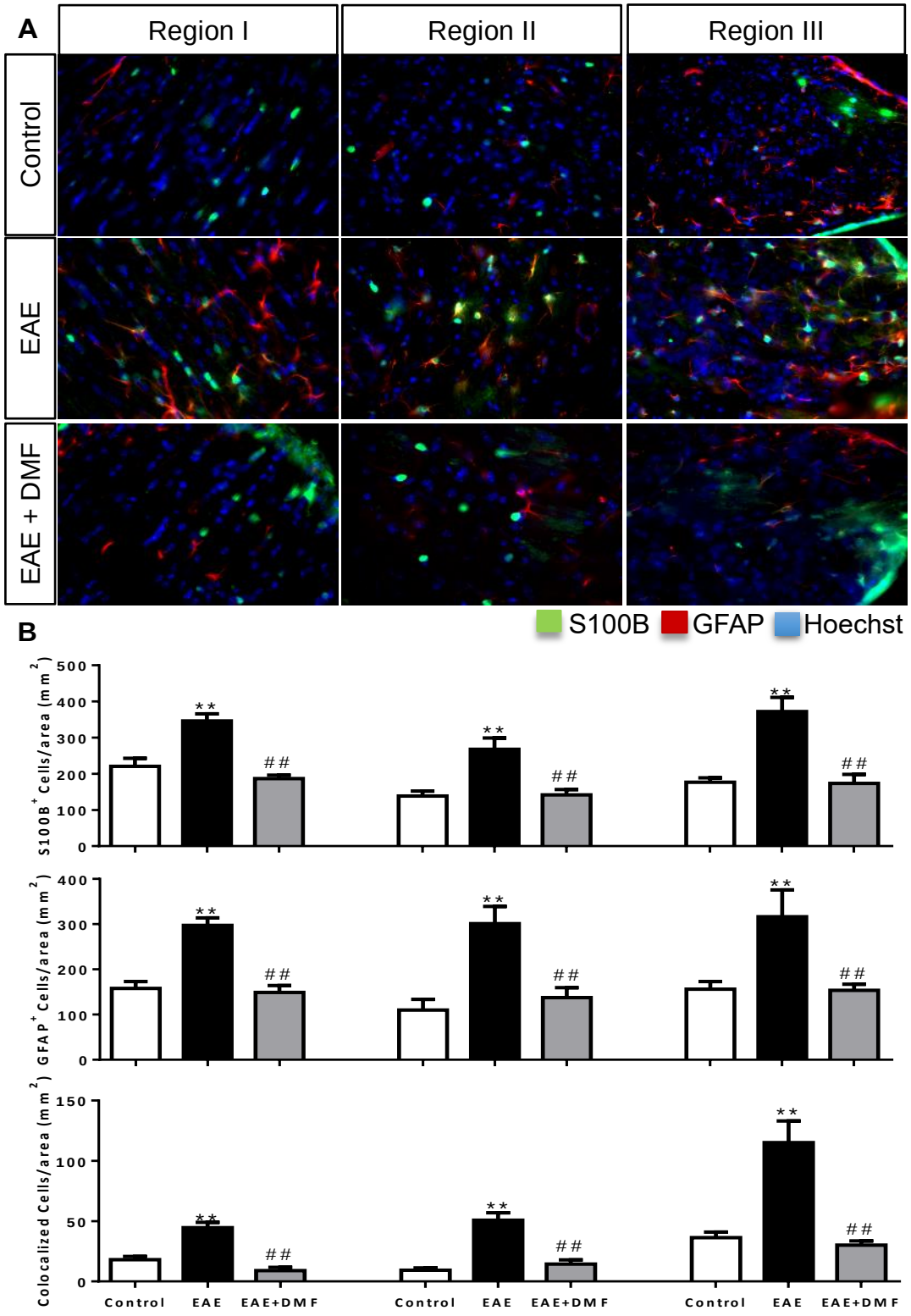

Figure III. 8 – EAE-induced animals have a high expression of S100B and GFAP that is prevented by DMF treatment. A. Representative images of brain slides of animals non-treated (Control), induced for EAE or induced for EAE and treated with dimethyl fumarate (DMF) during the chronic phase of the disease (EAE + DMF), immunostained for S100B (green) and GFAP (red) (Magnification: 40x). **B.** Graph bars represent the density of S100B-, GFAP-, or S100B/GFAP-positive cells per area (mm²) evaluated in the different regions: (I) fimbria (II) internal capsule and (III) perivascular zone. **P<0.01 vs. Control, and ##P<0.01 vs. EAE.

shown in Figure III. 9, EAE insult caused a marked overall increase of RAGE

(p<0.05) with a most marked expression in regions II and III (2.18-fold and 2.46-fold, p<0.05, respectively). The DMF treated group showed RAGE levels comparable to control ones, with a marked decreased over EAE ones (p<0.05).

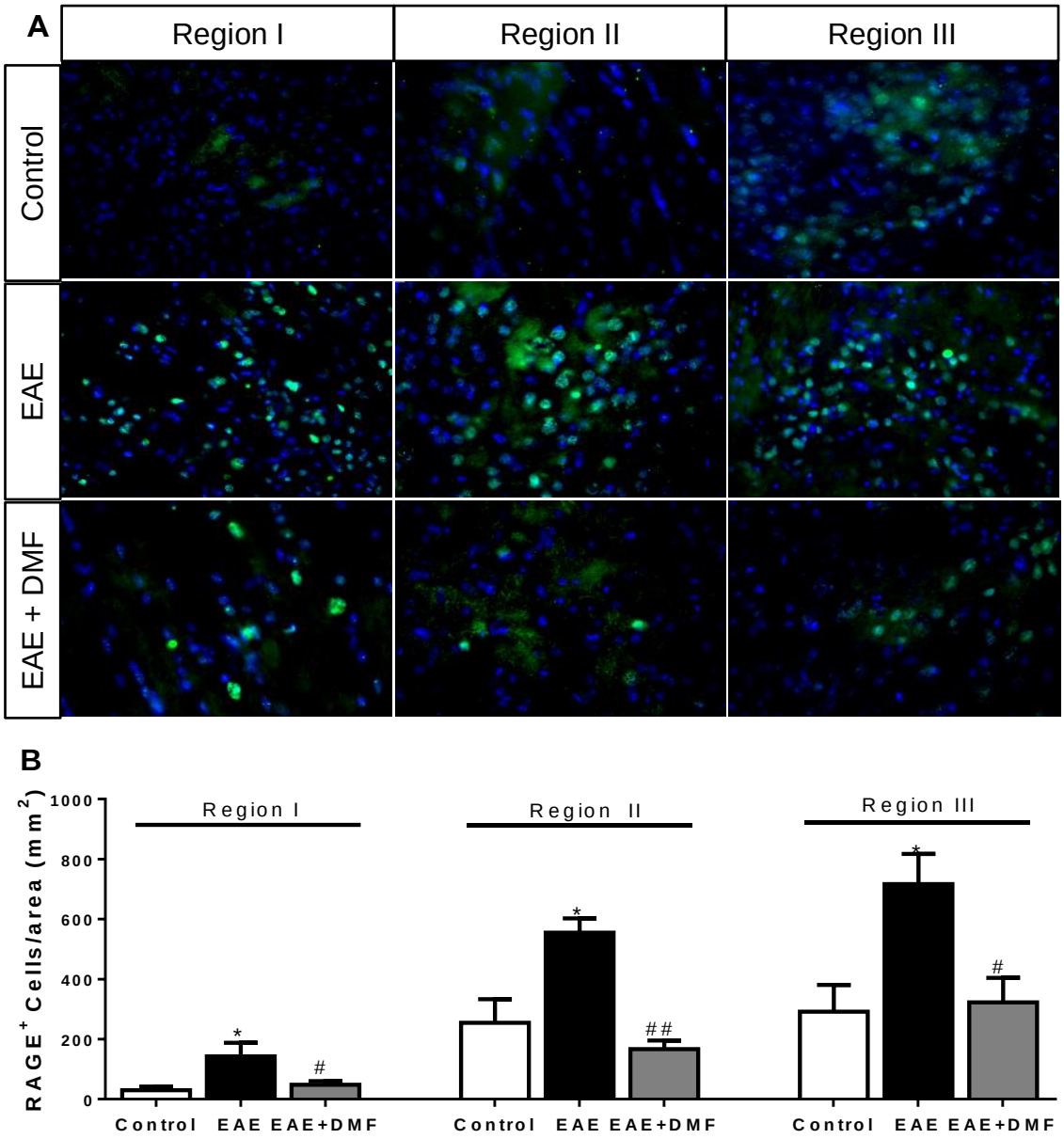

Figure III. 9 – Expression of RAGE is increased in EAE-induced animals but prevented upon DMF treatment. (A) Representative images of brain slides of animals non-treated (Control), induced for EAE or induced for EAE and treated with dimethyl fumarate (DMF) during the chronic phase of the disease (EAE + DMF), immunostained for RAGE (green) and stained with hoechst dye to detect nuclei (Magnification: 40x). **(B)** Graph bars represent the density of RAGE-positive cells per area (mm²) in the different regions : (I) fimbria (II) internal capsule and (III) perivascular zone. *p<0.05 vs. Control, and #P<0.05 and ##P<0.01 vs. EAE.

9. DMF attenuate inflammatory response triggered by EAE induce.

At high concentrations, S100B can exerts neurotoxic effects by microglial and astrocyte activation, leading the release of inflammatory and oxidative stress mediators, contributing to neuronal cell death (Sorci et al. 2010, Villarreal et al. 2014). Having observed the marked induction of S100B-RAGE axis we next assess the effect of high S100B levels on the inflammatory response in our EAE-induced mouse. For this end, we extracted total mRNA from slices and both TNF-α and HMGB1 expression were analyzed by qRealTime-PCR using specific primers. As shown in Figure III. 10, EAE insult caused a marked increase of TNF-α (1.73-fold, $p<0.01$) over control, that was completely prevented on the DMF treated group ($p<0.01$). On the other hand, HMGB1 expression showed a slight decrease (0.82-fold) that was even more marked when treated with DMF (0.36-fold).

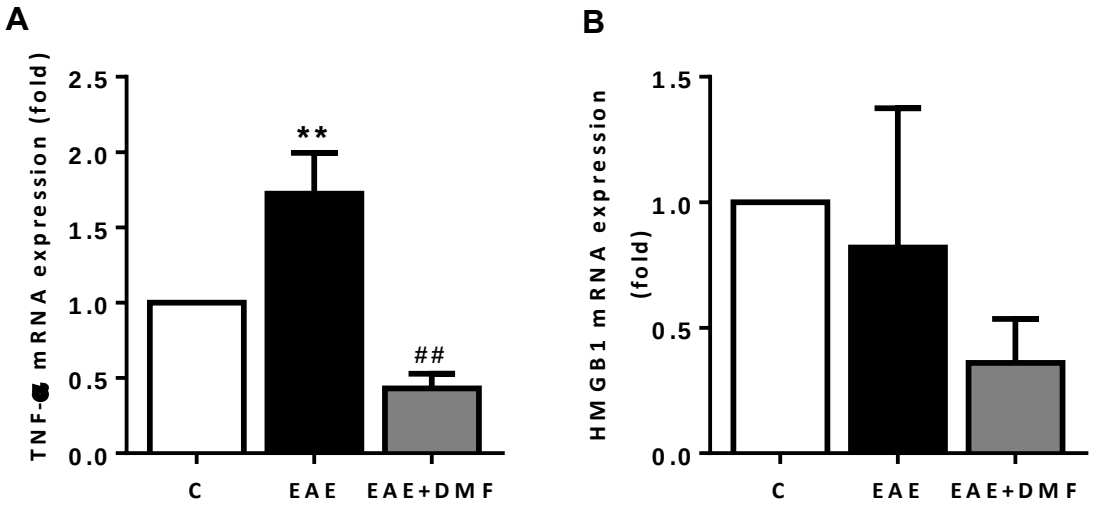

Figure III. 10 – Elevated S100B levels activate TNF-α and decrease HMGB1 gene expression in EAE-induced mouse, which are prevented and accentuated by DMF. Gene expression of TNF-α **(A)** and HMGB1 **(B)** was evaluated by qRealTime-PCR. Results are mean ± SEM. One-way ANOVA with Bonferroni multiple comparison test (**p<0.01 vs. control, and ##p<0.01 vs. EAE).

Overall, our results showed that EAE animals highly express S100B and RAGE, and DMF counteracts the expression of both proteins in parallel to the reduced loss of myelin fibers and the decrease of pro-inflammatory cytokines. This suggests that S100B-RAGE axis may be a new and more specific target to reduce brain injury and myelin deficits associated with perinatal inflammatory conditions.

IV. Discussion

The importance of S100B in neurodegenerative diseases has been recognized over the last years, being viewed as a therapeutic target in several disorders, and more recently also in MS by our group. The major hallmark of MS is the development of demyelinated lesions called sclerotic plaques, as a result of a sequence of events that involves reactive gliosis, inflammation, demyelination and remyelination until OPC depletion that culminates with axonal loss and neuronal degeneration (Milo and Miller 2014). Indeed, abnormal levels of S100B expression have been detected in both CSF and post-mortem plaques of MS patients, while it is known that S100B is a mediator of the inflammatory response in brain injury conditions (Donato et al. 2009, Barateiro et al. 2016). Thus, it becomes crucial to understand the role of excessive S100B in MS. In this thesis we intended to understand if by targeting the inflammatory molecule S100B we may prevent MS-associated pathogenesis. Firstly, we assayed a new S100B-targeting drug, pentamidine, in a demyelinating *ex vivo* model (Birgbauer et al. 2004) and next, we characterized the S100B-RAGE axis in a MS *in vivo* inflammatory model, the EAE.

We began by evaluating the production and expression of S100B after a demyelinating insult with LPC in the OCSC. Our results showed an overproduction and overexpression of S100B partially co-localized with GFAP-positive astrocytes. These data validate previous studies of our group using the same *ex vivo* model of demyelination where we observed the expression and production of S100B (Barateiro et al. 2016). According to the current data of all cell types of CNS, astrocytes have the highest expression rate of S100B, being

the major producers of S100B in the course of demyelination (Shashoua et al. 1984, Donato 2001). Interestingly, we observed that by adding pentamidine, a specific S100B inhibitor, to the incubation media in the presence of LPC, the expression of S100B partially decrease, suggesting that S100B is controlling its own expression. These results are in line with a previous report, showing that repression of S100B expression in astrocytoma cell line GL15, interferes with their ability to re-express the protein for a relatively long time which translates into long lasting effects on cell shape changes (Brozzi et al. 2009). However, when incubated alone without any LPC insult, pentamidine at high concentration (5 µg/mL) seems to partially increase S100B expression. These results possibly suggest a toxic effect of the higher concentration of pentamidine that results in a reactive response with S100B expression.

Relatively to S100B receptors, we observed that RAGE mRNA is more expressed than TRL4 mRNA during LPC insult, while in the presence of pentamidine is decreases to levels close to the control. In comparison, 0.5 µg/mL pentamidine appears to have a better effect on this reduction than a higher concentration corroborating the potential toxic effect for higher pentamidine doses. S100B release might be dependent on the presence and activation of RAGE on the cell surface, and with this interaction S100B might stimulate its own release via RAGE engagement (Perrone et al. 2008, Donato et al. 2009, Sorci et al. 2013). Taking this into account our results suggest that pentamidine may have a possible role in sequestering extracellular S100B, preventing it from interacting with RAGE receptor, decreasing therefore its own expression, although we may not exclude a direct pentamidine effect at the intracellular level. So, upon pentamidine treatment, low extracellular S100B is not able to promote the activation of astrocytes and microglia in a RAGE-dependent manner, as previous reported (Bianchi et al. 2007, Brozzi et al. 2009), decreasing also the expression of this receptor.

Similar to previous reports (Allt et al. 1988, Birgbauer et al. 2004), the concentration of LPC and the length of the stimulus we used on this work, effectively induced demyelination, demonstrated by a reduction in the number of myelinated fibers. Curiously, we observed that by adding pentamidine to the incubation media, in the presence of LPC, myelin sheaths damage was partially prevented. This demyelination is often accompanied by axonal lesions, which

largely account for patient disability (Woodruff and Franklin 1999, Jean et al. 2002), likewise we observed a decrease in the percentage of neurofilaments caused by LPC toxicity. Curiously when treated with pentamidine, we observed that both the myelinated fibers and the percentage of neurofilaments were closer to the control values. Our results suggest that with lower extracellular S100B, demyelination is partially prevented and low damage is observed on neurofilaments. To note, the higher concentrations of pentamidine, when added alone, had a negative and deleterious effect on the myelin sheaths and consequently on neurofilaments suggesting once more a possible toxic dose.

Additionally, we observed a marked decrease on the number of mature OL following LPC injury. Demyelinating events in MS are usually followed by spontaneous remyelination, leading to a total or partial clinical remission of the symptomatology. This remyelination involves the recruitment of resident OPC to the injured area, which proliferate and maturate into mature OL (Franklin and Ffrench-Constant 2008, Patel and Klein 2011). This activation of OPC includes changes in cell shape and size becoming more intensely labelled with NG2 (Levine and Reynolds 1999). In accordance, our results showed a greater number of NG2+ cells after LPC incubation relative to the control, suggesting an initiation of remyelination following 48h of initial LPC insult. Interestingly, treatment with pentamidine partially decreased the levels of NG2+ OPC when compared to LPC-treated group in parallel with an increase of the levels of MBP+ cells that were impaired upon LPC treatment. These results suggest that sequestering S100B in the extracellular media by pentamidine, partially prevents the death of mature OL cells and therefore less OPC need to be recruited and activated to compensate that loss. However, as protection was not completely effective, mature OL still need to be restored. This can explain the reason why OPC continue to have a high number over non-treated control OCSC. Additionally, it is known that S100 is necessary for OPC differentiation into mature OL (Deloulme et al. 2004). As so, it is possible that the presence of small levels of S100B that are not sequestered by pentamidine may be promoting OPC proliferation for consequently differentiation and remyelination. Therefore, additional studies should be performed for more extended periods of culture in order to analyze the remyelination potential of pentamidine treatment.

IV. Discussion

MS pathophysiology holds an inflammatory background, since during demyelination there is an astrocytic and microglial activation with an exacerbated production of inflammatory cytokines and chemokines (Mosser 2003, Sospedra and Martin 2005). S100B is mainly released by astrocytes and its expression is regulated by several factors including TNF-α and IL-1β (Guerra et al. 2011). High S100B levels are reported to increase proinflammatory cytokines release (Donato et al. 2013). The alarmin HMGB1, a cytokine involved in the initiation of inflammatory response, is upregulated in both MS lesions and rodent EAE lesions (Andersson et al. 2008). Our results showed a significant increase in the expression of first-line cytokines HMGB1, TNF-α and IL-1β upon a demyelinated insult, in parallel to an increased S100B expression as previously reported by us (Barateiro et al. 2016). Moreover, our data here also showed that blocking S100B with pentamidine, we were able to prevent these cytokines increase. Once activated microglia are the main cells that release these cytokines, these results suggest that microglia may shift from a pro-inflammatory phenotype to a less inflammatory one. The expression of the anti-inflammatory cytokine IL-10, as we expected, showed a tendency to remain highly expressed when compared to control levels indicating that the anti-inflammatory response is still maintained following pentamidine co-incubation. Interestingly, our results showed an increase in the pleiotropic IL-6 mRNA expression upon LPC that remained higher than control values even upon co-incubation with pentamidine. Despite the large number of studies reporting the role of IL-6 in the pathology of MS, there is still great controversy. Several studies investigated the expression of IL-6 in MS lesions and it was observed a peak expression in inactive demyelinated lesions, suggesting a protective role of this cytokine in myelin repair (Schonrock et al. 2000). This beneficial effect on demyelination has also been observed in several experimental models including the Theiler's murine encephalomyelitis (TMEV), where recombinant human IL-6 reduced demyelination and promoted remyelination (Rodriguez et al. 1994); or the EAE model, where IL-6, modulated by Oncostatin M showed a role in tissue repair (Wallace et al. 1999). In contrast, several studies reported an increase in IL-6 mRNA expression in cerebrospinal fluid (CSF) and plasma of patients with MS, showing that this cytokine is involved in MS progression (Frei et al. 1991, Navikas et al. 1996). It has also been reported that IL-6-deficient mice revealed absence of demyelination and inflammation after

EAE induction (Eugster et al. 1998). Our results shown that IL-6 is markedly released following LPC stimulus, but the fact that it is increased after treatment with pentamidine unlike other proinflammatory cytokines, shows that IL-6 may be playing a more anti-inflammatory and protective role in myelin repair.

Collectively, these results suggest that pentamidine is able to prevent demyelination and decrease the exacerbated production of inflammatory factors, indicating a beneficial effect in the prevention of demyelinating/inflammatory pathogenesis.

As previously mentioned, EAE is a Th cell-mediated autoimmune disease model characterized by T-cell and monocyte infiltration in the CNS associated with local inflammation, demyelination and neurodegeneration (Mathew et al. 2013). According, we observed from luxol stained slides a marked demyelination in EAE-induced animals, further supported by a reduced gene expression of OPC and mature OL markers as well as of synaptic markers (e.g. PSD-95 and synapthophysin). These results corroborate that the chronic EAE model lead to long term oligodendrogenesis failure accompanied by loss of synaptic markers. In this study we decided to use the inflammatory EAE model to characterize the S100B/RAGE axis in an *in vivo* model that mimics MS, for future assay pentamidine as a new therapeutic strategy MS pathogenesis.

Oral DMF has been recently approved for treating patients with relapsing MS (Fox et al. 2012, Gold et al. 2012, Bar-Or et al. 2013), yet, the mode of action of this drug is still not fully understood. Several studies suggested that DMF can downregulate T cell and myeloid cell proinflammatory responses (Longbrake et al. 2016, Michell-Robinson et al. 2016), while others demonstrated that DMF particularly targets proinflammatory mature B cell subsets (Li et al. 2017). Our results, as expected, showed a considerable reduction on damaged myelin sheaths, corroborated with an increase of OPC marker and mature OL marker expression. Relative to the expression of synaptic markers, our results shown that EAE-induced animals treated with DMF increased their expression to values close to the controls ones, suggesting that this drug is able to successfully protect myelin sheaths, promote oligodendrogenesis and recover of synaptic function. Nevertheless, since no changes were observed in animal's clinical score but only in learning tests, it is possible that these effects occur in specific regions. Further studies should be performed by treating EAE animals with DMF prior to the

clinical score peak to attest if DMF could prevent the manifestation or extent of the observed chronic lesions.

Reactive gliosis has long been associated with MS pathophysiology (Compston and Coles 2008) and is characterized by the hypertrophy and proliferation of microglial cells and astrocytes. Reactive astrocytes in particular, in addition to hypertrophy shows gene regulation changes including the upregulation of GFAP (Sofroniew and Vinters 2010). It is widely described in the literature that EAE-induced demyelination increases astrocytosis (Liedtke et al. 1998, Brambilla et al. 2009, Voskuhl et al. 2009). In agreement, our results show a marked overall increase of astrocytic activation caused by EAE insult, observed by the morphological change to a more amoeboid form with the reduction of cell extension length. As expected, DMF decrease this activation to values close to que control ones. As mentioned above, astrocytes are the main cells that secrete S100B when a demyelinated insult occurs (Donato et al. 2009). Accordingly, we observed high levels of S100B co-localized with astrocytes on the EAE-induced animals that decreased close to the control following co-treatment with DMF. High concentration of extracellular S100B exerts neurotoxic effects via astrocytic and microglial activation, that will consequently secrete pro-inflammatory factors that may inhibit OPC proliferation and maturation, contributing to neuronal cell death following demyelination in MS (Sorci et al. 2010). In our work, the marked decrease of astrocytes under demyelinating conditions in the presence of DMF also reduced S100B levels. At low concentration S100B act as a signal molecule and can enhance astrocytic proliferation (Selinfreund et al. 1991, Reali et al. 2005, Zhang et al. 2011), and under non-inflammatory environment, astrocytes are able to respond to demyelination by producing growth factors that increase OPC activation (Blakemore et al. 2003). Taking this into account, our data suggest that the beneficial DMF effect on the demyelination observed in EAE may be involving the S100B-RAGE axis.

S100B act as a signaling molecule through engagement of the RAGE receptor, in turn RAGE is upregulated by extracellular S100B and potentiate the induction of pro-inflammatory genes such as TNF-α and HMGB1 creating an inflammatory loop (Donato et al. 2013). In our data, we observed that overall increase of S100B and RAGE expression in EAE-induced animals is accompanied by an increased gene expression of TNF-α, possibly suggesting

that S100B-RAGE axis may have a role on the creation of an inflammatory milieu in this experimental model. Interestingly, treatment with DMF reverse almost completely this inflammatory expression. Curiously, HMGB1, was not upregulated, as occurred in our previous results in the *ex vivo* model. These results suggest that in the EAE model inflammation signaling may be under TNF-α, rather than HMGB1, regulation. Since HMGB1 can also compete with S100B for RAGE binding with consequent increased production and release of cytokines (Sims et al. 2010), the higher amount of S100B when compared to HMGB1 gene expression in parallel to increased RAGE suggest that in the present *in vivo* EAE model the S100B-RAGE axis may be more relevant.

Concluding Remarks

With this thesis we demonstrated, using an *ex vivo* OCSC model, that LPC effectively induced demyelination, through reduction of myelin fibers and neurofilaments, in the presence of S100B and RAGE overproduction and marked pro-inflammatory cytokines gene expression. On the other hand, by using pentamidine, we were able to prevent this demyelination by a blockade of the pathological S100B levels. Moreover, we showed that pentamidine also prevent the exacerbated expression of pro-inflammatory mediators reducing myelination damage and/or favoring remyelination. Collectively, these results strongly suggest that pentamidine is able to act upon S100B and prevent the detrimental effects of this protein when present in excessive supra-physiological levels.

In our EAE study, we demonstrated that EAE induced animals have effectively damaged myelin sheaths through reduction of gene expression of myelin protein and synaptic markers. Furthermore, we showed a marked increase in S100B and RAGE expression, as well as gene expression of pro-inflammatory TNF-α, suggesting that in this animal model S100B-RAGE axis may be active. Therefore, targeting S100B-RAGE axis may be a new and more specific therapeutic strategy to prevent demyelination and exacerbated expression of inflammatory factors.

Further studies on *in vivo* MS models will better clarify whether targeting of S100B-RAGE axis can be a good therapeutic strategy to reduce damage and increase recovery in MS.

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